Soluble urokinase plasminogen activator receptor in venous ulcers

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Declaration:

I hereby declare that the work in this thesis is my own and contains no work previously published elsewhere. All experiments were performed at the Academic department of surgery, St Thomas' Hospital, London, UK.

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Table of Contents

Abstract:

Compression therapy remains the only treatment option for venous ulcers with <100% success. A better understanding of the mechanisms regulating venous ulcer healing might give rise to better treatments.

Urokinsae plasminogen activator receptor (uPAR), a GPI anchored 3-domain protein exists either attached to cell wall or in soluble forms (suPARI-III, suPARI & suPARII-III fragments). Cleavage of uPAR results in a number of non-proteolytic functions that may be important in wound healing. Our aim was to determine whether suPAR and its fragments are present in the environment of venous ulcers and whether this receptor has a role in ulcer-healing.

Ulcer exudates and ulcer tissue biopsies were obtained from patients with confirmed venous leg ulcers. Venous ulcers that healed within 6months of compression therapy were classified as healers (H) while those that did not heal in this time were defined as non-healers (NH). Time resolved fluorescence immunoassays (TR-FIA) were validated for measurement of suPAR fragments. TR-FIAs specifically detected their antigens in wound exudates, but not in tissue homogenates. All forms of suPAR fragments were detected within venous ulcer exudates. Levels of all 3 suPAR fragments were significantly higher in exudates obtained from H compared to NH. ELISA analysis of uPA and plasminogen acitvator inhibitor (PAI-1) antigen revealed that levels were similar in ulcer exudates and tissue homogenates from H and NH.

Treatment of scratched keratinocyte cultures with exudates from H resulted in a greater coverage of the scratch compared with NH. Depletion of suPAR from exudates obtained from H resulted in cell death.

Results from this study have shown the presence of suPAR fragments within venous ulcers with higher presence of suPAR fragments in H compared to NH. This data suggests a role of suPAR and its fragments in ulcer healing, although the precise mechanisms involved remain to be identified. Measurement of suPAR fragments may provide a prognostic indicator and a therapeutic target for the treatment of venous ulcers.

Abbreviations:

ABPI: Ankle brachial pressure index

AWF: Acute wound fluids

CEAP: Clinical;Etiological;Anatomical;Pathological

CT: Computed tomography

CVI: Chronic venous insufficiency

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EVAR: Endovascular aortic aneurysm repair

EVLA: Endovenous laser ablation

FCS: Fetal calf serum

fMLP: Formyl-Met-Leu-Phe

FPRL1: Formyl peptide receptor 1

GPCR: G protein coupled receptor

HEK-a: Adult human epidermal keratinocytes

IL-1: Interlukin 1

LDLR: Low density lipoprotein receptor

LSV: Long saphenous vein

MMP: Matrix metalloproteinases

MRI: Magnetic resonance imaging

PAI-1: Plasminogen activator inhibitor – 1

PAI-2: Plasminogen activator inhibitor – 2

PAI-3: Plasminogen activator inhibitor – 3

PDGF: Platelet derived growth factor

PTFE: Polytetrafluoroethylene

RCT: Randomized controlled trial

RFA: Radiofrequency ablation

RPMI: Roswell Park Memorial Institue medium

SEPS: Subfascial endoscopic perforator surgery

SFJ: Saphenofemoral junction

SPJ: Saphenopopliteal junction

SSV: Short saphenous vein

suPAR: Soluble urokinase plasminogen activator receptor

TGFβ: Transforming growth factor beta

THP-1: Human monocytic leukemia cell line

TIMP: Tissue inhibitor of metalloproteinases

tPA: Tissue plasminogen activator

TR-FIA: Time resolved fluorescence immuno assay

uPA: Urokinase plasminogen activator

uPAR: Urokinase plasminogen activator receptor

V-ACT: Venous axial transposition of compentent tributary

VEGF: Vascular endothelial growth factor

Figures:

Tables:

Chapter 1: Introduction

1.1 Definition and historical background of venous ulceration

Ulceration is a loss of epithelial lining of an organ. When ulceration occurs on the lower limb skin secondary to an underlying venous pathology it can be defined as a venous leg ulcer. Venous ulcers are commonly seen on the lower medial aspect of leg also known as the gaiter area.

From the history of leg ulcers it is clear that although our understanding of the condition has improved, we are still far from finding the exact cause of ulceration and its cure. Venous leg ulcers were first described more than 2000 years ago by Hippocrates, who treated them with compression to express "evil homours"[1]. We learn about the impact of gravity on the development of venous leg ulcers by the 12th century saint of Forli in Italy, St Peregrine who never sat down for 30 years. He even slept while standing and therefore developed varicose veins and subsequently a leg ulcer[2]. By the 18th century it was thought that these leg ulcers were often chronic, not yielding to most of the treatments and they were due to an "underlying fault of blood"[3].Within the past few decades we have been able to establish that these ulcers are caused by chronic venous hypertension but how the venous hypertension leads to ulceration remains unclear[4]. A number of theories have been postulated to explain the relationship between venous hypertension and ulceration.

1.2 Theories for the cause of ulceration

Venous stasis Homans in 1917, was the first to describe stagnant blood as a cause of hypoxia leading to ulceration[5]. He suggested that patients with ineffective deep vein valves would have a "stagnant" column of blood leading to lack of nutrition and tissue hypoxia leading to ulceration. The theory of venous stasis was nullified by the work done by Blalock, who showed a higer oxygen concentration in blood obtained from patients with venous ulcers[6, 7].

Arteriovenous shunting The confirmation of higher oxygen content in venous blood of lower limbs from patients with venous ulcers stimulated the theory of "arteriovenous shunting". Shunting of blood in ulcerated limbs was first suggested by Holling et al in 1938, but Pratt in 1949 was the first to suggest the presence of arteriovenous shunts beneath the lower limb skin in patients with venous ulcers[2, 8]. In 1953 Piulacks and Vidal Barraquer supported this theory by showing a higher oxygen content and faster circulation time in limbs of patients with venous insufficiency. According to this theory artriovenous shunts lead to anaemic anoxia leading to ulceration of lower limbs. The findings of a study using microspheres suggests that there are no pathological shunts in patients with venous ulcers and therefore demolished the theory of arteriovenous shunts[9].

The fibrin cuff In 1982 Browse and Burnand postulated the fibrin cuff theory[6]. They suggested the extravasation of fibrinogen from the capillaries as a result of high venous pressure followed by polymerisation of fibrinogen into insoluble fibrin. The initial work done for this hypothesis showed increase

capillary permeability and escape of fibrinogen after eleveation of venous pressure in the hind limb of a dog. It was further postulated that due to the inadequate fibrinolytic activity, the fibrin deposited around capillaries resulted in a barrier to oxygen and other nutrients to the skin. This was supported by studies that showed histological finidings of fibrin cuffs[10]. The first question about the validity of this theory was raised by the same group when a randomized controlled trial using oral stanozolol (a profibrinolytic agent) did not result in better healing of venous ulcers[11]. In another study use of local fibrinolytic agent tPA also failed to dissolved the pericapillary fibrin[12]. The fibrin cuff theory has therefore lead to the confirmation of pericapillary fibrin deposition but the role of these fibrin cuffs in leading to ulceration could not be proven

White cell trapping According to this theory, propsed by the Middlesex group, white cells, which are larger than red blood cells, can block the capillaries resulting in decreased capillary perfusion pressure. Increased accumulation of white cells during prolonged venous stasis may therefore lead to local ischaemia leading to ulceration[13] [14]. These trapped white cells can also release toxic metabolites resulting in capillary damage and increased permeability for larger macromolecules like fibrin. Further studies attempting to prove this hypothesis failed to demonstrate capillary occlusion by white cells and therefore could not link hypoxia with occlusion of capillaries by white cells[15].

Growth factor trapping Another theory about the cause of venous ulceration was proposed by Falanga. This theory of venous ulceration proposes

that leaked macromolecules e.g. fibrin and $\alpha 2$ marcoglobulins trap growth factors that are required for tissue integrity and repair[16]. Just like the other theories, it is difficult to prove the cause and effect relationship of the trapped growth factors and venous ulceration [16-18]

These uproven theories about venous ulcercation demonstrate our lack of knowledge about mechanisms leading to venous ulceration. This limited knowledge is reflected on the lack of efficacious treatments options other than compression bandaging that is currently the mainstay of ulcer treatment.

1.3 Epidemiology and impact on population

About 1% of all adults will suffer from leg ulceration at some stage in their life with venous leg ulcers accounting for >70% of these ulcers[19]. In the latest and first global epidemiological study (The Vein Consult Program) the incidence of relatively less severe form of chronic venous disease (CEAP class C1-C3) was higher in females except for CEAP class C0 that was more prevalent in males. The more severe form of disease (C4-C6) was equal between the two gender groups[20].

The cost to treat venous disease (ranging from oedema to active ulcers) in UK was calculated to be >£600 million per year in 1992[21]. In addition to the cost of treating venous ulcers, there is also a high impact on quality of life as a result of pain and disability. Compression therapy is the main treatment for these ulcers and results in 70-80% healing within 6 months[22, 23]. If curative treatment is not undertaken re-ulceration remains a major problem with 25-55% of patients having recurrence of ulcers within 2 years[19, 24].

1.4 Pathophysiology of venous ulceration

Impaired return of blood from the lower limb results in venous insufficiency, which can be defined as a failure to reduce venous pressure with exercise[25]. During a non-pathological state the lower limb venous system functions to return blood from the legs towards the heart. An optimal calf pump with patent veins and competent venous valves aids the unidirectional flow. During walking or exercise both the pressure and volume in the lower limb venous system falls[25]. Venous pressure falls from 100 mm Hg to 22 mm Hg when a person starts walking (Fig 1). Chronic venous insufficiency (CVI) results in sustained venous pressure within the venous system referred as ambulatory venous hypertension. The reasons for sustained venous pressure can be failure of the calf pump, incompetent venous valves or obstruction of the venous outflow[25].

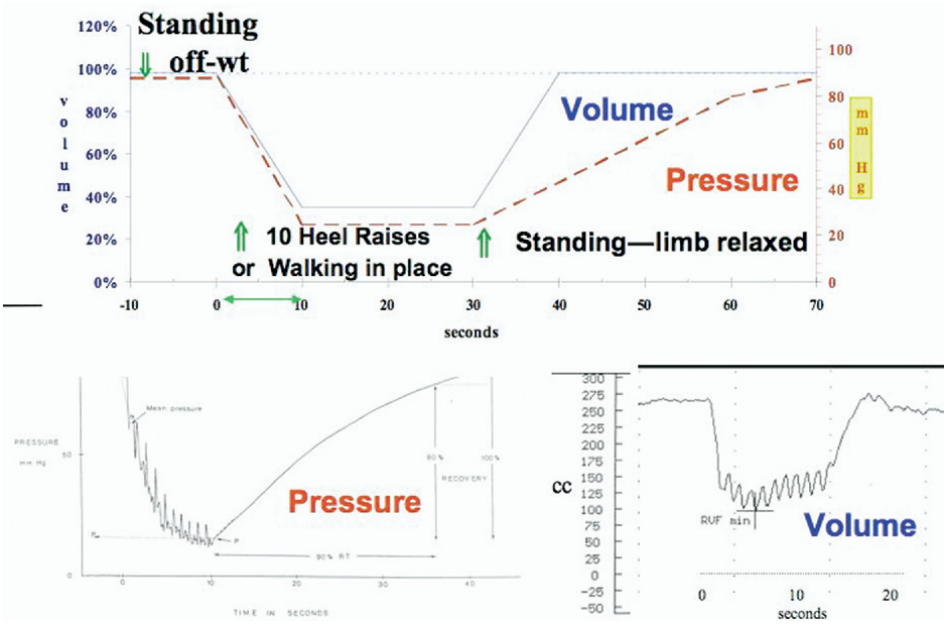


Fig 1 Venous pressure and volume changes[25]. The lower limb venous system aided by the calf pump and functional valves assists in decreasing the pressure and blood volume upon walking.

1.4.1 Calf pump failure

Muscle groups in the foot, calf and thigh all act as pumps to aid venous return, but the most important and efficient is the calf pump, which generates pressures of up to 200mm Hg during contraction[25]. Structured exercise strengthens the calf pump and improves venous haemodynamics[26]. Failure of the calf muscles to pump the venous blood against gravity is a major cause of ambulatory venous hypertension. The calf pump can fail as a result of muscle weakness from disuse atrophy that is commonly seen in elderly patients. Any major injury to the calf muscle or a neurological disease e.g. motor neuron disease can result in calf pump failure. Joint pathology e.g. arthritis especially effecting the ankle joint will also lead to an impaired calf pump

1.4.2 Incompetent venous valves

Valves exist in both the superficial and deep venous systems, in addition to the junctions between the two systems. Incompetent valves at the junction of superficial and deep veins (sapheno-femoral junction:SFJ and sapheno-popliteal juction;SPJ) result in blood flowing against the normal pattern of flow (i.e. superficial to deep). This results in transmission of high venous pressure into the superficial veins. The exact mechanisms leading to valvular dysfunction remain unclear. Incompetent venous valves along with weak dilated vein walls are seen in patients with CVI[27]. Recent evidence suggests weakening of the vein wall to be the initial pathology [27, 28]. As the weakness progresses towards the valves it renders them incompetent. The weakening and subsequent dilatation of the vein wall has been related to abnormal elastin metabolism[29] and dysregulation of collagen synthesis[30].

There is now evidence to suggest a strong genetic association in primary valve failure. Normal development of venous and lymphatic valves appears to be dependent on the FOXC2 gene as mutations in this gene have been implicated in the development of primary valve failure[31]

1.4.3 Outflow obstruction

Obstruction to the venous flow in the deep veins can also result in venous hypertension leading to CVI. Most common form of outflow obstruction is the formation of a thrombus within the deep veins which often starts in the calf

veins[32]. Ulceration develops in 5% of patients after deep vein thrombosis[33], which arises when one or more of the triad of factors - reduced blood flow (stasis), damage to the vessel wall, and hypercoagulability are present[34]. Thrombosis in the deep venous system can result in valve damage (though valve damage may also be the initial cause of thrombosis) and development of post thrombotic syndrome[32], which is defined as the presence of chronic venous symptoms and/or signs resulting from deep vein thrombosis[35]. Post thrombotic syndrome can present with a variety of sings and symptoms ranging from leg swelling to ulceration[36].

1.5 Investigations to establish a venous cause of ulceration

In order to look for causes of a leg ulcer a detailed clinical examination is important. Once venous etiology is established the CEAP classification is helpful in establishing the extent of venous leg pathology. The CEAP (C-clinical manifestation, E-etiological factors, A-anatomic distribution, P-pathophysiology) has been modified since its first introduction in 1994[37].

Investigations that confirm the findings of clinical examination may start with the use of hand held Doppler that can be performed in clinic. Colour duplex, plethysmography and venography are the techniques available to investigate the venous system of lower limbs. Duplex scanning is sensitive and the most commonly used method of identifying venous pathology. It has overtaken plethysmography and venography as it is non-invasive, relatively quick and inexpensive[38, 39]. A combination of duplex scanning and venous filling index

(VFI) obtained by air plethysmography is considered highly sensitive and specific[40]. Venography can be used to assess the deep veins. It is warranted for patients who have an unclear diagnosis or in whom duplex remains inconclusive.

Improvement in ultrasound technology in the form of intravascular ultrasound has shown significantly higher sensitivity when compared to transfemoral venography for detection of iliac vein stenosis[41]. Improved Computed tomography (CT) and Magnetic Resonance Imaging (MRI) has also enhanced the sensitivity to identify thrombosis in the deep venous system[39].

1.6 Treatment options

Surgical correction of the underlying pathology has been shown to prevent recurrence of ulcers but combining it with compression bandaging does not improve ulcer healing[42]. The main treatment for venous leg ulcers is compression bandaging. A variety of multi-layered bandages are in use for the treatment of venous ulcers. The amount of pressure exerted by each type is different more so because of the subjective differences in the pressure applied when putting on the bandage. The most commonly used are the four-layer and three-layer paste regimen. The healing rate of ulcers is not influenced by the use of different layers of bandaging[43].

In addition to compression bandaging a number of other treatments have been used for venous leg ulcers but none of them on their own have shown any significant improvement in ulcer healing. These include use of platelet rich fibrin matrix[44], ultrasound[45] and hyperbaric oxygen[46, 47]. In addition

some treatment options have been used as an adjunct to compression bandaging. These include electromagnetic therapy[48], electrical therapy[49] and honey[50]. However the evidence for the use of these adjuncts is still weak and these are not generally used in practice. The pharmacological adjuncts used along with compression therapy have shown a better outcome with both pentoxifylline[51] and microinonized flavonoids[52] showing improved healing rates when used along with compression therapy.

Once ulcers have healed a number of interventions can be undertaken to address the underlying pathology and thereby prevent recurrence. A brief description of the treatments that may be used to treat lower limb venous pathology in order to prevent venous ulcer recurrence is given below

1.6.1 Compression stockings

Compression stockings exert external pressure that is greater at the ankle and then reduces proximally. In cases where surgical correction of the underlying pathology is not possible graduated compression stockings are the only solution to prevent ulcer recurrence. It improves the function of the venous pump[53]. The compression can thereby provide relief of symptoms and improve the venous haemodynamics[54]. Patients are usually given Class II UK standard compression stockings that would give a pressure of 18-24 mm of mercury. Other types of class II stockings (European & US) give a different pressure. A compression of 10-30 mm of Hg is considered to be effective for the management of symptomatic varicose veins, whereas compression of 30-40 mm of Hg is effective for healing ulcers and preventing progression of post

thrombotic syndrome[55, 56]. The above knee stockings provide no advantage over the below knee stockings for prevention of recurrence[57]. The level of patient compliance with the compression stockings especially during hot weather is often not satisfactory.

1.6.2 Treatment for superficial venous system pathology

A simplified chart of the available treatment options for superficial venous system pathology is shown in Fig 2.

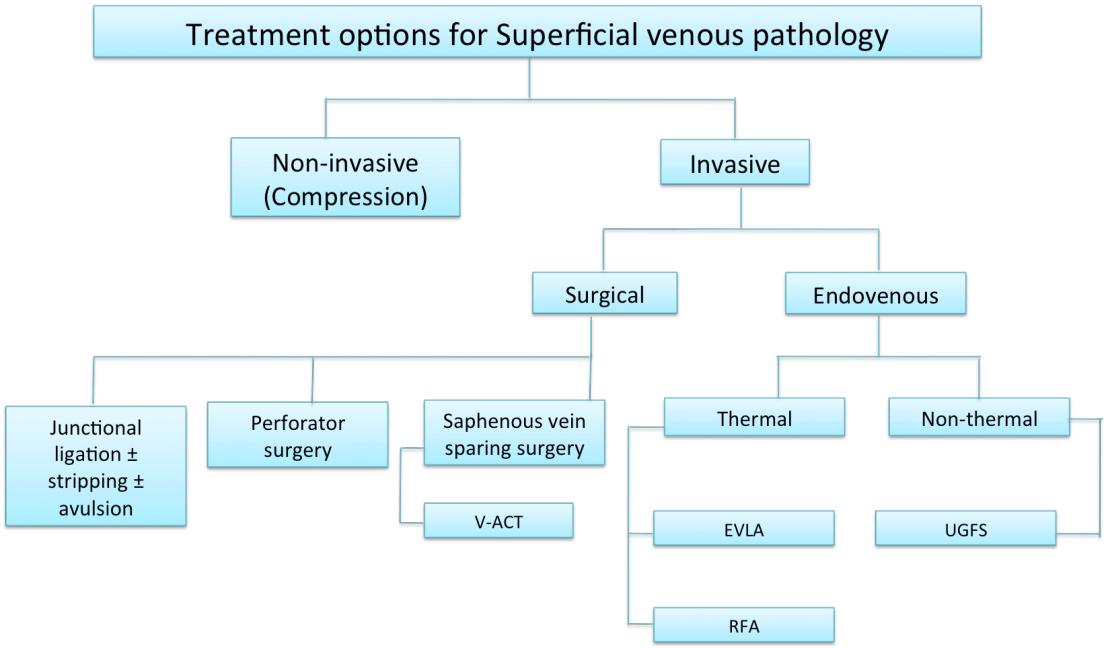


Fig 2: Flow chart for the treatments that may be used to treat reflux within the superficial venous system. V-ACT: Valvuloplasty with Axial transposition of Competent Tributary, EVLA: Endo Venous Laser Ablation, RFA: Radiofrequency Ablation, UGFS: Ultrasound Guided Foam Sclerotherapy

Prior to the currently available endovenous techniques the standard treatment for varicose veins was junctional ligation (SFJ or SPJ) ± stripping of the vein (LSV or SSV) ± stab avulsions of varicosities.

In cryostripping the only difference is that after ligation of the SFJ instead of a plastic stripper a metallic cryoprobe is passed down to LSV. The LSV is frozen at a temperature of – 85 °C (expansion of liquid nitrogen oxide in the cryoprobe) and then stripped. A RCT showed inferior results of cryostripping compared with conventional surgery with significantly higher residual LSV in the cryostripping group[58].

Surgery specifically aimed to correct the haemodynamic abnormality due to incompetent perforators includes subfacial endoscopic perforator surgery (SEPS). The use of SEPS has not shown any significant improvement in the quality of life or ulcer recurrence when compared with standard LSV stirpping[59, 60].

A desire to spare the LSV resulted in the development of techniques such as axial transposition of competent tributary (V-ACT). In V-ACT valve cusps are repaired from outside the vein wall followed by transposition of pre-operatively identified competent vein tributary[61]. Difficult learning curve and a strict pre-operative patient selection (having repairable valves) limited the use of this technique.

Amongst the more popular treatment options for the treatment of varicose veins is the endovenous thermal ablation that includes radiofrequency ablation

(RFA) and endovenous laser ablation (EVLA). Both techniques are less invasive and have been shown to be as effective as open surgery[62].

Another endovenous technique is ultrasound guided foam sclerotherapy (UGFS). The overall efficacy (occlusion of diseased vein and recurrence or development of new veins) of foam sclerotherapy in treating varicose veins is reported at 84%[63]. According to the most recent NICE guidelines on the use of foam sclerotherapy there is insufficient evidence to reliably compare foam sclerotherapy with surgery due to the lack of long term follow up trials comparing all the variables[63].

1.6.3 Treatment for deep venous system pathology

A simplified chart for treatment options of the deep venous system pathology is shown in Fig 3

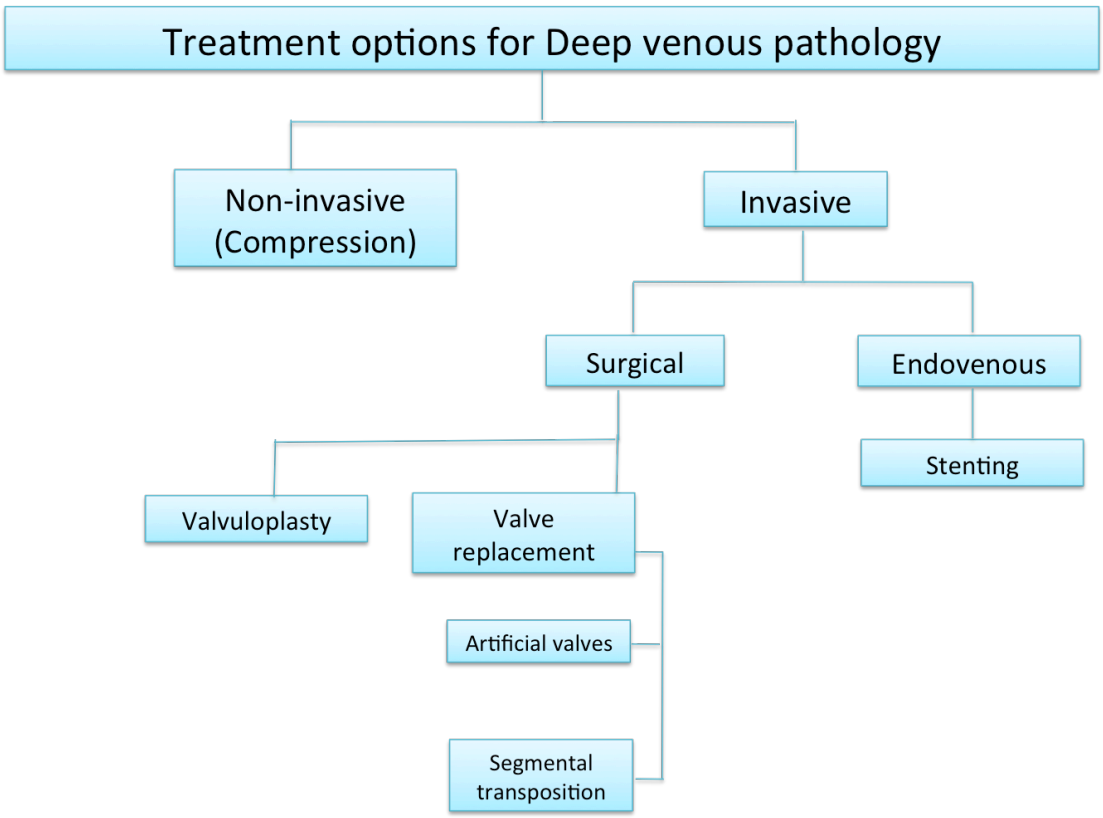


Fig 3: Flow chart of treatment options for deep venous pathology

Patients with post thrombotic syndrome or deep venous valve incompetence are only considered for surgical treatments once non-invasive methods (compression therapy) have failed. Even then surgical corrections of deep venous incompetence are only undertaken at specialist centers. Techniques include internal valvuloplasty (where the valve is repaired after making a longitudinal incision over the vein)[64-66], external valvulopasty (where sutures are placed externally)[66] and angioscopic repair (where the transluminal repair is made after passing an angioscope into the vein)[66, 67]. The use of external valves (Venocuff™) for the SFJ has reported a 90% competence of SFJ at 44 months[68]. This procedure however is only suitable for mild varicose veins and due to its strict preoperative selection criteria it

never gained popularity. Choice of technique, valve reconstruction in post thrombotic limbs and multiple valve reconstructions remain areas of controversy in the field of deep vein valvuloplasty[66]

In cases where valve repair is not possible (e.g. in post thrombotic syndrome) artificial venous valves[69] and segmental venous transposition has been used[70]. Improvement in endovascular techniques has allowed use of stents for occluded iliofemoral veins. Raju et al reported a case series where stenting in 38 limbs with iliofemoral occlusion showed primary patency of 49% at 24 months with 70% of patients having complete pain relief[71]

1.7 Pathophysiology of normal wound healing

Non-healing venous ulcers are types of wounds with an arrested healing process. Understanding normal wound healing helps in assessing the pathological processes that may be contributing towards a non-healing venous ulceration.

Wound healing is a complex process that can be divided into different phases: hemostasis, inflammation, proliferation/re-epithelialization and remodelling. However, none of the phases of wound healing is a distinct process and they are often synchronously merged into each other, with the same wound going through more than one phase at one particular time. At the microscopic level these phases are represented by a number of coordinated cellular and extracellular processes including chemotaxis, proteolysis, proliferation, angiogenesis, cell migration and cell adhesion.

The inflammatory phase starts with ***hemostasis*** and may last from 2 days to 2 weeks with neutrophils and macrophages being the main orchestrators of the inflammatory response. Platelet aggregation during this phase causes thrombin formation leading to the activation of the fibrin clot[72, 73]. Platelets release various growth factors and inflammatory mediators[74]. The redness and swelling observed at the time of injury is the result of arterial dilatation and increased vascular permeability secondary to the release of histamine from mast cells[73]. Fibroblasts that migrate in the wound during this early phase start secreting proteases and growth factors[74]. After the initial 2-3 days neutrophils are replaced by circulating monocytes which after embedding in the extracellular matrix (ECM) differentiate into tissue macrophages[75]. Monocytes/macrophages play a vital role during the inflammatory phase as they initiate angiogenesis and formation of granulation tissue that continues during the proliferative phase.

Cells involved in the ***proliferative phase*** are keratinocytes and monocytes/macrophages in addition to fibroblasts. The ECM takes over the central role during this stage leading to the formation of granulation tissue[73]. This new ECM laid down primarily by fibroblasts[74], consists mainly of collagen type III and fibronectin and is crucial for the progression of wound healing as it allows the cells to interact with each other and with other ECM proteins providing a transport system for the cytokines and growth factors. Activation of uPA after binding to uPAR converts plasminogen, a serine protease into its active form plasmin. Activated plasmin stimulates ECM degradation not only by direct degradation of basement membrane glycoproteins but also by

activating pro-MMPs to MMPs[76]. Proliferation and angiogenesis continues in the proliferative phase during the laying down of a new ECM and the keratinocytes at the edges of the wound start migrating towards the center of the wound. Arrest of keratinocyte migration is often seen in chronic non-healing wounds[77, 78].

Remodelling leads to continued wound contraction and maturation of the ECM and eventually complete wound healing. Wound contraction which overlaps both the proliferative and remodeling phase is mainly dependent upon the myofibroblasts as these cells containing high concentration of actinomyosin can contract along lines of contraction[73]. This is supported by the release of cytokines like EGF that promote keratinocyte migration and re-epithelialization[74]. In murine models expression of uPAR has been associated with tissue remodeling[79]. One of the important aspects of the phase of remodeling is the replacement of type III collagen by type I collage mediated by MMPs (matrix metalloproteinases), TIMPS (tissue inhibitors of matrix proteinases)[80] and TGFβ1[74]. Expression of at least two members of the MMP family (MMP-2 and MMP-9) is associated with an increase in active form of uPA[81]

1.8 Cellular and molecular abnormalities in venous ulcer healing

1.8.1 Keratinocyte migration

True wound healing is judged by the ability of the epidermal keratinocytes to re-epithelialise and cover the wound[82]. Similarly in venous ulcer healing epidermal keratinocytes play a critical role. It has been shown that the keratinocytes at the edge of the venous ulcer are able to proliferate but unable

to migrate towards the centre of the wound[77, 83]. The formation of a new and healthy epithelium is dependent on proliferation and migration of keratinocytes to cover the epithelial defect of the wound[82]. Migration of keratinocytes in turn is dependent upon a number of factors including ECM, hypoxia, growth factors and protease activity [82, 84, 85].

During normal wound healing the keratinocytes at the edge of a wound are stimulated after a lag of 24-48 hrs[86]. They start changing their morphology, lose their hemidesmosomes and desmosomes and prepare for interaction with the ECM. As there is no basement membrane over the wound the plasma membrane of the keratinocytes starts synthesizing a provisional matrix in the wound bed, while keeping contact with the basement membrane collagens (type IV and VII) and interstitial collagens (type I, III and VI)[87]. This also results in the release of various growth factors and cytokines into the ECM by the keratinocytes and the surrounding fibroblasts. Whether ECM or the growth factors are independently important for the initiation and augmentation of migration of keratinocytes has been a matter of debate[87, 88]. Both the ECM and growth factors have important roles in the migration of keratinocytes, with the latter stimulating keratinocyte migration in the presence of a pro-motility ECM matrix[89]. In the initial phase of wound healing type III collagen and fibronectin are deposited in the wound bed and later on type III collagen is replaced by type I collagen. This type I collagen is considered indispensable for a pro-motility ECM.[90-92], and helps the migration of keratinocytes.

The question that still remains unanswered is why in some venous ulcers keratinocytes proliferate and migrate to re-epithelialise the wound, but fail to do that in others.

1.8.2 Imbalanced angiogenesis

Angiogenesis is an important part of normal wound healing. Right from the early inflammatory phase of healing various angiogenic regulators e.g. vascular endothelial growth factor (VEGF), fibroblast growth factor 1 (FGF-1) and FGF-2 are released into the ECM[93]. Fibroblast growth factor-2 can stimulate endothelial cells to express uPA, uPAR and procollagenases to promote degradation of ECM, that is a pre-requisite for effective angiogenesis[94]

As opposed to normal skin, chronic venous ulcers have an increased amount of VEGF, a potent regulator of angiogenesis in healing wounds. Despite increased amounts of VEGF the angiogeneic drive in venous ulcers remains suboptimal[95, 96]. The presence of an inhibitor of angiogenesis in non-healing venous ulcers still remains to be elucidated

1.8.3 Hypoxia

The presence and relevance of hypoxia in venous ulcers has also been a matter of debate. Some studies show that there is hypoxia within ulcers[97, 98] while others do not[99]. Falanga et al have shown a variable oxygen tension around individual ulcers[100]

Most of the remodelling occurring due to hyopxia is controlled via hypoxia inducible factor-1 (HIF-1). Through a number of moleculer targets HIF-1 can regulate endothelial cell survival, migration and proliferation, thus influencing wound healing[101]. Earlier studies suggested increased keratinocyte migration during hypoxic conditions[102]. Later studies however suggested that hypoxia only stimulates the migration of young keratinocytes and not keratinocytes from elderly patients[103]. A definite answer to how hypoxia/HIF-1 can regulate venous ulcer healing remains to be answered

1.8.4 Imbalanced proteolysis

During tissue remodelling cells move through tissue barriers. This movement is facilitated by regulation of specific proteinases that digest local extracellular matrix (ECM) proteins without causing significant damage. Degradation and remodelling of ECM is therefore an important step in wound healing[104].

Matrix metalloproteinases (MMPs) are a growing family of endopeptidases that degrade structural proteins[105]. They are classified into collagenases, gelatinases, matrilysins and membrane type MMPs[80]. The optimal level of proteolytic activity is maintained through the concomitant expression of tissue inhibitors of metalloproteinases (TIMPs). So far 4 types of TIMP and 23 types of MMPs have been discorvered[80]. Imbalanced proteolytic activity mediated by MMPs has been strongly associated in the pathogenesis of venous ulcers[106, 107]. Most studies suggest increased activity of MMP-1, MMP-2, MMP-3 and MMP-9 activity in venous ulcers and lipodermatosclerotic skin[105, 108, 109], but this is dependent upon presence of other factors such as tumour necrosis factor alpha (TNF-α)[110] the activity of other proteases such as uPA[111], and

metalloproteinases like MMP-10 which is co-expressed with MMP-2 at the epithelial border of venous ulcers[105]. Levels of both uPA and MMP-9 have been shown to be significantly higher in the chronic wound exudates when compared with exudates from acute wounds, suggesting uncontrolled proteolysis within the environment of chronic wounds[81]. The ECM can also modulate the secretion of MMPs as suggested by the inhibitory effect of native fibronectin on proMMP-9 secretion when compared with fragmented fibronectin[110]. A decrease in the levels of MMPs following compression therapy of venous ulcers has been observed suggesting an association between ulcer healing and MMPs[108].

Leak of plasminogen, acting as a persistent substrate for uPA also results in a potent pro-proteolytic enviornment in the ulcer[81]. The plasmin that is generated from plasminogen can either directly degrade the ECM and basement membrane glycoproteins or indirectly cause this through activation of MMPs[76, 80]. Plasmin production is augmented by the action of plasminogen activators and inhibited by plasminogen activator inhibitors (PAI), which are all present within the environment of a venous ulcer[112].

1.9 Plasminogen activation system

Components of the plasminogen system play an important role during different stages of normal wound healing, with plasminogen gene knockout mice having poor wound healing when compared to mice with combined uPA and tPA knockouts[113]. The presence of various components of the plasminogen system within the environment of a venous ulcer begs the question as to which

component of this system has a major role to play in the healing of venous leg ulcers.

The plasminogen system functions to remove pathological fibrin clots within the body. Important components of this system are[114]

- Zymogen
    - Plasminogen that can be converted to plasmin
- Activators
    - Tissue plasminogen activator (tPA)
    - Urokinase plasminogen activator (uPA)
- Inhibitors
    - α_2-macroglobulin
    - α_2-plasmin inhibitor
    - Plasminogen activator inhibitor 1
    - Plasminogen activator inhibitor 2
    - Plasminogen activator inhibitor 3
    - Protease nexin
- Receptors
    - Urokinase plasminogen activator receptor inhibitor (uPAR)
    - Low-density lipoprotein receptor (LDLR)

1.9.1 Plasminogen

Plasminogen is made up of 791 amino acids and is primarily synthesised in the liver. It can be found in plasma at a concentration of 1.5 μmol/l with a half-life of 2 days[114]. It is made up of a single protease domain and five homologous

kringle domains[115]. The first and fourth kringle domains impart high and low lysine binding affinity respectively. Based on the lysine binding affinity plasminogen mediates its interactions with fibrin, cell surface receptors and its circulating inhibitor α_2-plasmin inhibitor[114]. The primary function of plasminogen is to cleave the insoluble fibrin polymers after its conversion to the active form. Plasminogen is converted to its active form plasmin after cleavage of a single peptide bond by the action of one of the two plasminogen activators, either tissue-type of plasminogen activator (tPA) or urokinase-type of plasminogen activator (uPA)[76]. In the absence of fibrin tPA is a weak activator of plasminogen[116] whereas uPA is an effective activator of plasminogen both in the presence or absence of fibrin[117]

1.9.2 Plasmin

Plasmin, a two-chain molecule, is the activated form of plasminogen. It contains the classic serine protease catalytic triad (His-Asp-Ser) but it is a non-selective serine protienase that can break down fibrin in addition to other proteins[114]. In addition plasmin can also activate other proteases and growth factors (TGF-β)[111, 118]. One of its important roles in wound healing is the direct degradation of the extracellular matrix[76]. It can indirectly effect matrix degradation by the activation of MMPs[119]. Plasmin is inhibited by alpha2 macroglobulin and alpha2 antiplasmin also known as the α_2-plasmin inhibitor[114, 120].

1.9.3 Urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA)

These activators have similar activities but are two enzymes derived from expression of two different genes[121, 122]. The gene coding for uPA is present on chromosome 10 and the gene encoding for tPA is on chromosome 8[76]. Urokinase plasminogen activator is mainly an extra-vascular activator of plasminogen and its main function is to generate pericellular plasmin activity[123], while tPA's main function is to remove cross linked fibrin that forms in the vascular tree[124]. Due to this reason tPA has been used in clinical situations to dissolve clots

Urokinase plasminogen activator is a serine protease secreted in a pro-active (pro-uPA) form having a molecular weight of 54kDa[125]. It is synthsized in kidney, lung, keratinocytes and endothelial cells[126]. Pro-uPA is a single chain molecule (sc-uPA) that undergoes conversion to the active two-chain form (tc-uPA), after cleavage of peptide bonds proteases including plasmin and kallikrein and angiogenic factor VEGF[127-129]. The active tc-uPA is usually referred to as uPA. Tc-uPA exists either in high molecular form (54kD) or in a low molecular form (33kD) with the difference of a 135 amino terminal fragment between the two forms[117]. Both forms are active but whether there is a specific function for the cleaved low molecular form still remains to be elucidated. The N terminal A chain contains the epidermal growth factor like (EGF) and kringle domains, while the carboxy terminal B chain contains the protease domain. The EGF domain of uPA mediates its binding with the uPA receptor (uPAR)[130]. Although the pro-uPA form has minimal peptidase activity yet it can

preferentially activate fibrin bound plasminogen, presumably because plasminogen undergoes a conformational change when it binds to fibrin making it more susceptible to the action of pro-uPA[131]. Plasmin activates the single chain pro-uPA converting it into the two-chain form, which has a greater protease activity than the single chain pro-uPA. The rate at which tc-uPA can cleave plasminogen is 200 times higher than the single chain pro-uPA[132]. Plasma and urine concentration of uPA is 5-10μg/l[126]

The second plasminogen activator tPA is also secreted as a single chain form and is converted to the active two chain form by cleavage of a peptide bond by plasmin, kallikrein or coagulation factor Xa[126]. Plasma concentration of tPA is 5-10μg/l and it is expressed by a number of cells including endothelial cells and keratinocytes[126]. Single chain tPA contains a finger domain, growth factor domain, kringle domain and the protease domain[76]. In addition to binding to fibrin and activating the fibrinolytic system, tPA can also bind to thrombospondin, fibronectin and the endothelial cell surface which also enhances its enzymetic activity[76]

A number of factors, relevant to wound healing regulate post-transcriptional expression of uPA and tPA. These include epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factor beta (TGFβ), Interlukin 1 (IL-1) and glucocorticoids[76]. The presence of a specific cell bound uPA receptor (uPAR) localizes its activity to the pericellular space. This is an important function of uPA, as it localises pericellular proteolysis to the leading edge of migratory cells[117, 133]

1.9.4 Urokinase plasminogen activator receptor (uPAR)

A specific extracellular uPA receptor (uPAR) was identified in 1985[134]. It is a glycosyl-phosphatidylinositol (GPI) anchored protein made up of approximately 283 amino acids with no intracellular component[123, 135]. This receptor is made up of three domains, DI, DII and DIII[123]. Although the binding site of uPA on uPAR(CD87) is on DI[136], yet the complete receptor with its characteristic close proximity of DI and DIII is necessary for ligand binding[137].

The interaction of uPA with uPAR increases the ability of uPA to convert plasminogen into plasmin. This is due to the fact that the binding of uPA with uPAR slows down the plasmin mediated degradation of uPA, thereby allowing more conversion of plasminogen into plasmin[138]. Due to the GPI anchor uPAR can have variable positions on the plasma membrane depending upon the state of the cell, so that on a resting cell uPAR is evenly distributed whereas on a migrating cell it can cluster onto the leading edge[139]. uPAR has been identified on a number of cell types including fibroblasts, monocytes, keratinocytes and endothelial progenitors[140-143].

Patients with paroxysmal nocturnal haemoglobinuria have a soluble form of the uPAR receptor (suPAR) which was not attached to the cell membrane[144]. Subsequent studies showed the existence of suPAR in various pathological states including rheumatic and neurological disease and in various cancers[145]. Proteolytic cleavage between DI and DII can result in the formation of two separate fragments, suPAR-I and suPAR II-III. Interestingly the cleavage of uPAR between D1 and D2 may be achieved by uPA[146] in addition to

other proteases[147]. If the cleavage between DI and DII occurs while the receptor is attached to the cell membrane suPAR-I is released and uPAR II-III remains attached to the cell membrane. Cleavage of uPAR by uPA is much faster on the cell surface compared to in solution[148]. In addition the cleavage of uPAR is also dependent upon its glycosylation pattern as the heavily glycosylated uPAR (in ARO thryorid cancer cell line) is resistant to cleavage by uPA. In comparison a less glycosylated uPAR (in TAD-2 thyroid cancer cell line) is easily cleaved by uPA. The cleavage of GPI anchor can result in shedding of both the complete receptor suPAR-I-III and the fragments containing domains DI and DII-III (Fig 4)[148-150].

The soluble forms of uPAR are found in a number of biological fluids including plasma, cystic fluid and ascitic fluid[145, 148]. uPAR and its fragments are also elevated in a number of inflammatory disorders, during angiogenesis and in certain cancers (e.g. gastric and prostate cancers)[148, 151].

The internalisation and regulation of uPAR requires binding of this receptor with uPA-PAI-1. The uPAR-uPA-PAI-1 complex associates with the LDRL and is then endocytosed into the cell. This internalisation may be clathrin dependent or independent (clathrin is a protein present on the cell surface, that is involved in endocytosis and exocytosis)[152].

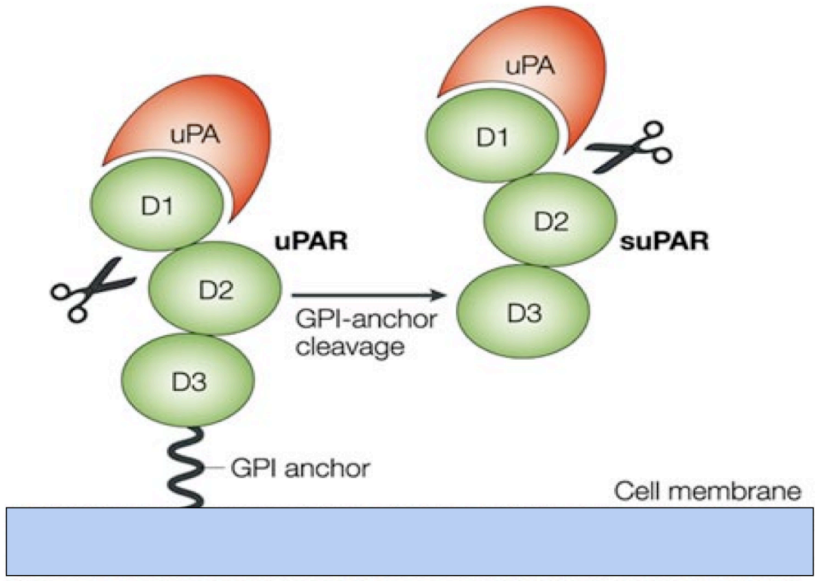


Fig 4. uPAR variants[151]. Cleavage of the GPI anchor can result in liberation of the whole molecule as suPARI-III whereas cleavage between DI and DII will result in the formation of two separate fragments DI and DII-III

1.10 Mechanisms of cell signalling by uPAR

Urokinase plasminogen activator receptor is an extracellular receptor which in addition to its proteolytic function of activating plasminogen into plasmin exhibits additional "extra-proteolytic" functions e.g. cell adhesion, cell migration, chemotaxis and cell differentiation[153-156]. Being a purely extracellular molecule uPAR is dependent upon other transmembrane molecules to manifest its intracellular functions. At least three transmembrane molecules have been identified through which uPAR can manifest its extra-proteolytic functions. Given below is a brief description of these three mechanisms

1.10.1 Integrins

Interaction with intergrins is important for function of uPAR. The integrins are transmembrane heterodimers consisting of α and β subunits. In humans 18 α and 8 β subunits have been identified. Each subunit consists of a large extracellular region and smaller transmembrane and cytoplasmic regions[152]. Integrins can influence cell morphology, intracellular signalling and cellular migration by interacting with molecules that regulate cytoskeletal organization[151]. Much of the intracellular signalling resulting from the interaction of uPA and uPAR results from integrin mediated pathways. Both anti-uPAR and anti-integrin antibodies can block in vitro uPA dependent migration of a variety of cell lines. In addition the induction of in vivo transperitoneal migration of neutrophils appears to be dependent upon uPAR-integrin interaction[151]. Of the various integrins with which uPAR has partnered the interaction with β1 and β3 integrins has been analysed in detail and is therefore relatively better understood[157]. Binding of uPA to uPAR is required for signalling via β1 integrins. α5β1 is a fibronectin receptor where as α3β1 is a laminin receptor and both have been shown to affect cell signaling due to their interaction with uPAR[152]. The other important integrin involved in uPAR signaling is αvβ3, a vitronectin related integrin that can influence actin polymerization and cellular motility[152].

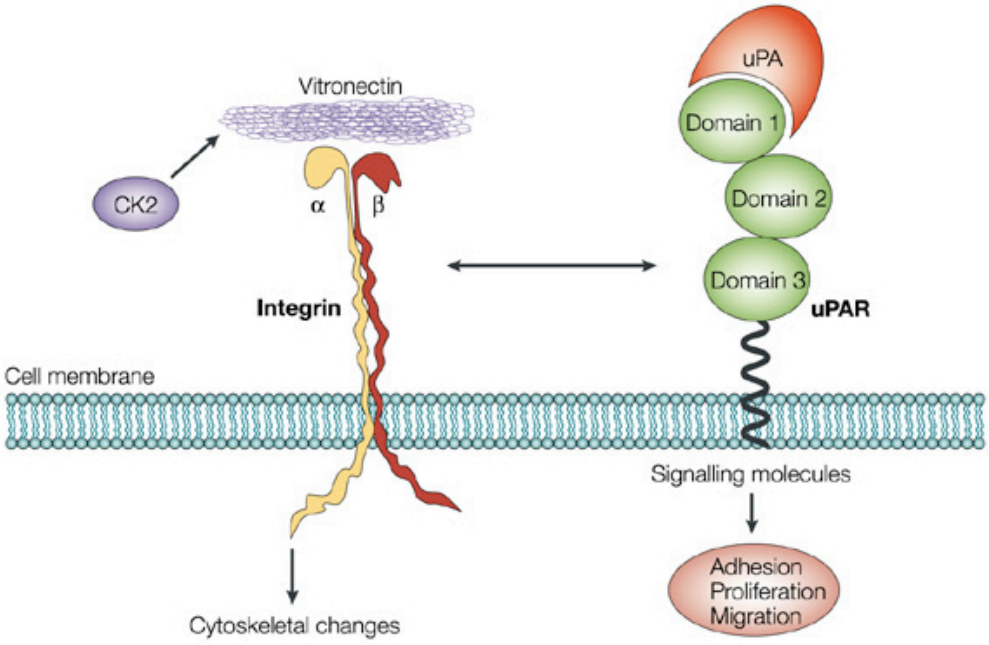


Fig 5. Interaction between uPAR and integrins in cell signaling[151]. uPAR an extracellular molecule affects cellular adhesion, proliferation and migration by interacting with transmembrane molecules like integrins. CK2= Casein kinase 2 which activates vitronectin

1.10.2 G protein coupled receptors (GPCR)

The other transmembrane molecules with which uPAR partners for intracellular signalling are the G (guanine nucleotide binding) protein coupled receptors (GPCR). Of all the GPCR receptors the formyl peptide receptor 1 (FPRL1), a homologue of fMLP (formyl-Met-Leu-Phe), has been investigated in more detail in respect to its interaction with uPAR. The chemotactic activity of uPA-uPAR is dependent on FPRL1 as cells lacking this transmembrane receptor do not respond to uPA or suPARII-III[151]

1.10.3 Caveolin and lipid rafts

Lipid rafts are part of the plasma membrane containing cholesterol, gangliosides and GPI anchored molecules e.g. uPAR. Caveoli are a type of lipid raft found in cell surface invaginations implicated in endocytosis[151, 158]. uPAR colocalizes with the lipid rafts (GM1 and GM3) in a resting cell but upon stimulation specific types of lipid rafts (GM3) having uPAR are stimulated and become localized to the functionally active part of the cell (e.g. the leading edge in a migratory cell). Therefore the subcellular localization of uPAR (either clustering at the leading edge or a more generalized distribution) determines the type of signaling response (e.g. migration versus proliferation) [159].

1.11 Role for uPAR in wound healing

The plasminogen system is an important regulatory mechanism involved in fibirnolysis. Components of this system have been identified in the environment of non-healing ulcers. However the plasminogen system has additional non-proteolytic functions that may have implications in venous ulcer healing. Most of these non-proteolytic functions are dependent upon the receptor uPAR, which therefore can have an important role in wound healing. Given below are some of the areas where uPAR mediated cellular responses may be involved in wound healing

1.11.1 Cellular inflammatory response

Urokinase plasminogen activator receptor is increased in a number of inflammatory disorders including rheumatic disease, HIV and inflammatory renal diseases etc[151]. It may therefore influence the initial inflammatory phase of wound healing as uPAR has shown to induce chemotaxis of neutrophils and monocytes[160, 161]. In addition keratinocytes involved in wound healing have shown an increased expression of uPAR[151]. Although the inflammatory potential of uPAR has not directly been observed in a wound environment, yet migration and adhesion of neutrophils is shown to be influenced by increased monocytes uPAR expression[160, 162].

1.11.2 Pericellular proteolysis

The urokinase receptor may have the maximum influence in wound healing during pericellular proteolysis which facilitates the removal of degraded matrix components making the ECM suitable for cell migration, cell adhesion and angiogenesis[163]. Plasmin generated from the action of uPA on plasminogen following binding to uPAR stimulates ECM degradation not only by direct degradation of basement membrane glycoproteins but also by activation of pro-MMPs to MMPs[76]. uPAR localises uPA over the cellular surface and thereby increases pericellular proteolysis[164], making the ECM more suitable for other cellular functions.

1.11.3 Cellular proliferation

Wound healing progresses from the edge of a healing wound that starts by keratinocyte proliferation followed by their migration towards the defect. Studies in uPAR gene knockout mice show that keratinocytes expressing uPAR proliferate much faster than those cells deficient in this receptor [165]

1.11.4 Cellular chemotaxis

The DII-III domain of both soluble and GPI anchored forms of uPAR is responsible for cellular chemotaxis. One of the first studies that showed the presence of a chemotactic peptiede at the linker region between DI and DII also suggested that this function of uPAR was mediated via the G coupled proteins[154]. The DII-III domain of suPAR may therefore have a potential role in inducing cellular chemotaxis during wound healing

1.11.5 Cell migration & adhesion

Variation in mechanical stresses may lead to different responses in healing wounds. Both the ECM and the cells within a wound respond to internal and external mechanical changes by exhibiting changes in the collagen fibers and cellular cytoskeleton[166, 167]. Interaction of uPA with intact uPAR containing the D1 domain can lead to cell migration independent of the proteolytic activity of uPA. This uPA dependent cell migration may be carried out via αVβ5 integrin[168] or GCRP[169]. In cleaved uPAR lacking the D1 domain, cell migration depends upon the binding of the chemotactic peptide SRSRY with N-

formyl peptide receptor (FPR) or FPRL1[170]. Cells expressing uPAR adhere to ECM by binding to VN and this interaction is necessary for the resulting cytoskeletal changes leading towards cell migration[171]. In instances where there is no direct interaction between uPAR and VN, uPA can lead to similar uPAR dependent cytoskeletal changes[172]. Increased cell-matrix adhesion is vital for healing as it stimulates myofibroblast differentiation and formation of stress fibers[173]. In the presence of adhesive stimuli keratinocytes from uPAR knock-out mice produced much reduced deposition of laminin 5 when compared with keratinocytes from wild type mice[165], signifying the importance of uPAR in adhesion induced expression and deposition of laminin 5, which is important for migration of keratinocytes.

Evidence for the role of uPAR in cellular adhesion and migration is supported by impaired neutrophil infiltration and reduced monocytes adhesion in uPAR deficient mice in response to Pseudomonas aeruginosa infection[160]. This however may only be a uPAR response during inflammation and not during wound repair. uPAR-VN adhesion and resulting cytoskeletal changes are enhanced [174, 175] by the presence of uPA. A possible explanation for this is the close proximity of the binding sites of uPA and VN on uPAR, with uPA binding providing stability for VN binding[175]

1.11.6 Angiogenesis

Abnormal angiogenesis is linked with poor wound healing[95]. Although uPAR has been implicated in tumour angiogenesis it may have an indirect or direct role in angiogenesis during wound healing. uPAR enhances pericellular

proteolysis and thus plays a role in ECM remodeling[164], which is an essential step of angiogenesis during healing. The direct effect of uPAR on angiogenesis is via its interaction with PAI-1. During resting cell state PAI-1 can block uPA-uPAR interaction and the subsequent integrin ($\alpha v\beta 3$ and/or $\alpha v\beta 5$) dependent migration and angiogenesis. However in active endothelial cells most of the uPA-uPAR complex is concentrated at the leading edge of the cell, which overcomes the inhibitory ability of PAI-1.

1.12 Hypothesis and rationale for the study

Although a number of studies have found uPA and uPAR within venous leg ulcers[107, 176], yet there are no reports about the presence of suPAR fragments within the environment of a venous ulcer. To date, there have only been 5 studies that have looked at the presence and potential role of the plasminogen system in venous ulceration.

The first study done at St Thomas' Hospital by Stacey et al.[177], found significantly higher levels of uPA in venous ulcers when compared to ischaemic ulcers. Tissue plasminogen activator (t-PA) was only detected in a small number of venous ulcers, suggesting that uPA was the major plasminogen activator in venous ulcers.

Herouy et al.[178] showed increased uPA and uPAR protein (centred around the capillaries) and increased uPA activity in venous ulcers compared to normal skin.

In a study by Weckroth et al. significantly higher levels of uPA protein was reported in keratinocytes and fibroblasts treated with venous ulcer exudate compared with cells treated with acute wound exudates[176].

In another study by Herouy significantly higher uPA (protein and activity) and uPAR (mRNA and protein) expression was found in lipodermatosclerosis skin compared with normal skin[107]. Like the previous studies these were again found to be located in the pericapillary region.

In another study Weckroth et al suggest that tPA may be more important than uPA in chronic ulcers healing[179]. Tissue plasminogen activator protein was found in all layers of 6/8 chronic venous ulcers, including stromal cells (fibroblasts and macrophages). It was only expressed in a few keratinocytes in acute wounds. Urokinase mRNA was expressed only in the wound edge keratinocytes and stromal cells of 6/8 venous ulcers and all acute wounds; with expression being higher in the acute wounds. It was again located in the pericapillary region.

None of these studies analyzed the suPAR fragments and the comparisons were between venous ulcers and normal skin or acute wounds. There was no comparison between healing and non-healing venous ulcers

1.12.1 Hypothesis

Soluble fragments of uPAR are present within the environment of venous leg ulcers.

1.12.2 Aims

1. To validate time resolved immunofluorescence assay for detection of soluble forms of uPAR in wound exudates and tissue homogenates.
2. To measure total and active uPA, and its main inhibitor, PAI-1 in venous leg ulcers.
3. To compare the levels of suPAR fragments and other components of plasminogen system in healing and non-healing venous ulcers
4. To determine whether suPAR fragments affect the migration of keratinocyte in vitro.

Chapter 2: Patient recruitment and general methods

2.1 Patient recruitment and sample collection

Ethical approval for the study was obtained from St Thomas' Ethics committee (Ref: 09/H0802/4). Potential participants were identified at the weekly leg ulcer clinic at St Thomas' Hospital. Patients aged >16years with leg ulcers, ankle brachial pressure index (ABPI) of >0.8, confirmed venous aetiology at venous duplex and starting compression therapy were included in the study. The exclusion criteria were ABPI ≤0.8, pregnancy, immunosuppressive therapy, age ≤16years or a non-venous ulcer. Patients who satisfied the recruitment criteria were informed about the study and a written consent was obtained. Blood plasma samples were obtained from ten patients only, as per instructions of the ethical committee.

Ulcer exudates and 6mm ulcer edge biopsies were obtained from each participant at start of compression therapy. Acute wound exudates were collected from the skin donor sites of the patients undergoing split skin grafting.

2.2 Collection of wound fluid

Patients were asked to attend the hospital in the morning. The ulcer was cleaned with normal saline and then covered with a transparent occlusive

dressing, Opsite® (Fig 1). Patients were then encouraged to drink and walk as much as possible, as walking resulted in a greater production of exudate. After 3-4 hrs ulcer exudate accumulated underneath the Opsite® was aspirated with a needle. The exudate was then centrifuged at 16,000g at 4^{o}C for 10mins and the supernatant aliquoted, were snap frozen and stored at -80˚C till needed.

Acute wound exudates were collected from underneath the Opsite® dressing placed over the donor site of patients undergoing split skin grafts for non healing venous ulcers. Acute wound exudates were processed similar to the chronic wound exudates

Fig 6: A typical venous ulcer covered with Opsite® dressing through which exudate was aspirated

2.3 Collection of ulcer tissue

A sample of ulcer tissue (6mm punch biopsy) was taken from the ulcer edge (including a rim of healthy skin) following injection of 1% lignocaine local anaesthetic, The collected tissue was snap frozen at -80˚C for biochemical analysis.

2.4 Collection of blood plasma

Blood (10ml) was collected from the superficial anticubital arm veins of ten patients using a 21gauge needle at the time of collection of ulcer exudates/tissue samples. The collected blood was centrifuged at 400g and 4˚C for 15mins. Plasma was aliquoted, snap frozen and stored at -80˚C.

2.5 Definition of healing and non-healing

The time required for a wound to heal varies from individual to individual depending upon a number of factors. There is no defined time period when a venous ulcer may be categorized as non-healing. Once a venous ulcer has not healed after conventional compression treatment it may be defined as a non-healing ulcer and other treatment options e.g. skin grafting may be considered. This usually depends on the overall reduction in the size of ulcer during regular inspections. For the purpose of our study we defined *healers* as those ulcers that healed within 6months of compression therapy, whereas those who did not heal within this time period were defined as *non-healers*.

All wound fluids obtained from venous ulcers are referred to as healing or non-healing ucler exudates. Fluids that were pooled or obtained from acute wounds are referred to as wound fluids.

2.6 Patient demographics

A total of thirty venous ulcer exudates were collected (n=9 healers and n=21 non-healers. Tissue biopsies were collected from 27 patients. Out of these 7 biopsies were from healers and 20 from non-healers. Age, sex and average ulcer size of the patients are given in table 1 and 2. There was no significant difference between the two groups from which wound exudates was collected. The only significant difference between the two groups from which tissue biopsies were taken was younger age of patients in healers. Acute wound fluids were obtained from 7 patients.

Table 1: Comparison of demographics and ulcer size between patients from whom wound exudates were collected. Ulcer size was expressed as the product of two largest diameters

	Healers	Non-Healers	P value
Total number	9	21	
Median age (yrs, range)	62 (36-79)	70 (40-92)	NS
Sex (M:F)	4:5	10:11	NS
Median ulcer size (cm^2 range)	16 (3-165)	31 (3-140)	NS

Table 2: Comparison of demographics and ucler size between patients from whom tissue biopsies was obtained. Ulcer size was expressed as the product of two largest diameters

	Healers	Non-Healers	P value
Total number	7	20	
Median age (yrs, range)	55 (36-79)	72 (36-88)	<0.05
Sex (M:F)	4:3	8:12	NS
Median ulcer size (cm^2 range)	8 (3-165)	14 (3-140)	NS

Chapter 3: Validation of suPAR ELISA in ulcer tissue and wound fluids

3.1 Introduction

Specific immunoassays for detection of soluble froms of uPAR (DI-III, DI, and DII-DIII) have been validated for human plasma samples[180] but not for human wound exudates or tissue homogenates from wound biopsies.

The aim of this part of the study was to validate time-resolved fluorescence immunoassays (TR-FIAs), used for plasma analysis of suPAR and its fragments, for measurement of these analytes in venous ulcer tissue and wound exudates. For this specificity, linearity, accuracy and intra-assay precision of the assay for wound exudates and tissue lysates was analysed.

3.2 Methods

3.2.1 Extraction of plasminogen components from tissue

Polytetrafluoroethylene (PTFE) cups and metal balls, used to pulverise tissue samples (Mikro Dismembrator, Sartorius), were equilibrated with liquid nitrogen. Ulcer tissue samples were weighed (whilst frozen) prior to being pulverized in the dismembrator at 3000rmp for 60secs. Extraction buffer (0.1 M Tris-HCl, pH 8.1, 1% Triton X-114, 10mM EDTA, 10μg/ml Aprotinin (the latter

added immediately prior to tissue extraction) was then added to the tissue powder (1ml per 100mg of tissue) and the suspension pulverised at 3000rmp for a further 60secs. The homogenate was pipetted from the cups into 1.5ml conical polypropylene tubes (Eppendorf) and centrifuged at 16,000g and 4^{o}Cfor 10mins. The supernatant was aliquoted, snap frozen in liquid nitrogen and stored at -80°C until assayed.

3.2.2 TR-FIAs of suPAR and its fragments in tissue lystaes and exudates

Pooled wound fluid and tissue homogenate were diluted in Delfia assay buffer (Perkin Elmer) 1:30 and 1:50 respectively. Each of the pool was analysed for selectivity, loss of linearity, inter-assay and intra-assay precision. During each assay a quality control citrate plasma (diluted 1/5) was also analysed. The primary and secondary antibodies used for the TR-FIAs were a gift from Dr Gunilla Hoya-Hansen[180]. Primary monoclonal antibody for TR-FIA1 and TR-FIA2 was R2, while the primary antibody used for TR-FIA3 was R5. The detection antibodies used were labelled with europium. For TR-FIA1 and TR-FIA3 the detection antibody used was Eu-R3, and the detection antibody for TR-FIA2 was Eu-R23[180]. Cross reactivity of TR-FIA3 for suPAR (I-III) was blocked by addition of peptide antagonist AE120[180]

TR-FIA1 measures suPARI-III; TR-FIA2 measures suPARI-III and suPARII-III; TR-FIA3 measures suPAR I. In order to get amount of suPARII-III results of TR-FIA1 are subtracted from TR-FIA2.

3.2.3 Depletion of suPAR

Samples diluted in Delfia assay buffer were depleted off suPAR using column chromatography. Separate columns were used for tissue homogenates and wound exudates. The steps used in depletion are as follows

1. A 1ml HiTrap Protein A HP column (GE Life sciences 17-0402) was washed with 20 ml of PBS.
2. Rabbit polyclonal anti-suPAR antibody (1ml) diluted in PBS to a concentration of 50μg/ml was added to the column. The eluate was collected and re-applied repeating the process a total of 5 times.
3. Column was again washed with 20ml of PBS.
4. Samples diluted in Delfia assay buffer were run through the column and eluate collected. The eluate was then re-applied to the column, repeating the process a total of 3times. The final collected eluate was the depleted sample.

3.2.4 Time resolved immunoassays

TR-FIA1 was used for detection of intact suPAR (I-III), TR-FIA2 for detection of suPAR (I-III) & suPAR (II-III) and TR-FIA3 for suPAR(I). Total suPAR (II-III) was calculated by subtracting the results of TR-FIA2 from TR-FIA1.

One volume of primary antibody was diluted in six volumes of a 0.01MHCl solution. After 5mins the acidified primary antibody was diluted in a 0.1M NaH_2PO_4 solution to get a final concentration of 1μg/ml for the primary antibody. This solution was used to coat the White Maxisorb (NUNC) fluorostrips (200 μl per well), which were left overnight at room temperature

after covering with plastic cover slips. The next day plates were washed twice with Delfia wash solution and blocked with a solution containing 50mmol NaH_2PO_4, 1g/L diazolidinyl urea, 60g/L sorbitol and 1g/L diethylenetriaminepentaacetic acid purified BSA (300µl per well). Plates were sealed and stored overnight at 4°C. Plates were emptied, dried in a fume hood for 3hrs and resealed.. The coated plates were stored for up to 4wks at 4°C prior to use. for the time resolved immunoassays.

Assay steps

1. Duplicates of standards and samples diluted in delfia assay buffer were added to the wells of the pre-coated plates (100µl per well). Standards were added at the following concentrations 10, 3.33, 1.11, 0.37, 0.123, 0.041, 0.014 and 0ng/ml (blank).
2. Plates were then incubated for 1hr at room temperature with shaking.
3. Plates were then washed 6times with Delfia wash buffer.
4. Europium labeled detection antibodies, diluted in Delfia assay buffer were added at concentration of 1µg/ml (100µl per well). For TR-FIA3 1µM AE120 was added along with the detection antibody.
5. Plates were again incubated at room temperature for 2hrs with shaking.
6. Plates were again washed 6 times with Delfia wash buffer.
7. Enhancement solution was then added (100µl per well) followed by shaking for 3mins.
8. Flourescence was measured using fluorometer (Victor, Perkin Elmer) with excitation and emission wavelengths set at 340nm, and 615nm respectively, with a 400µs delay.

3.2.5 Specificity, linearity, accuracy and intra-assay precision

Specificity: *Specificty* is the ability of the assay to differentiate suPAR fragments from other proteins in a sample. This was assessed by carrying out analysis of samples following depletion of the suPAR. Pooled wound fluids and tissue homogenates that were depleted off suPAR were analyzed together with the non-depleted pool of wound fluids and tissue homogenates using TR-FIA1, TR-FIA2 and TR-FIA3. Percentage cross-reactivity was calculated [(depleted sample/non-depleted sample)×100]. A cross-reactivity of ≤20% was considered an acceptable result.

Linearity: Assessment of linearity is the concentration at which the difference between the expected and calculated values exceeded 20%. This was determined by measuring suPAR and fragments in serial dilutions of pooled wound fluids and tissue homogenates (1:10 – 1:320). Pooled wound fluids and tissue homogenates were serially diluted to 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320. The serially diluted samples were analyzed using TR-FIA1, TR-FIA2 and TR-FIA3. The curve of serially diluted samples remaining parallel to the standard curve was considered an acceptable result.

Accuracy: *The accuracy* of an assay can be assessed by measuring the recovery of a known concentration of standard (spike) that is added to a sample. Pooled wound fluids and tissue homogenates with known concentration of suPAR were spiked with serially diluted known concentrations of standard. Percentage spike recovery was calculated using the formula [(Measured sample+spike/Expected sample+spike)×100]. Recovery of 100±20 was considered an acceptable result.

Intra-assay precision: The intra-assay precision is determined by analysing replicates of a sample and calculating the coefficient of variation (CVs). Ten samples from pooled wound fluids and tissue homogenates were analyzed at the same time using TR-FIA1, TR-FIA2 and TR-FIA3. Percentage coefficient of variation was calculated using formula [(Standard deviation/Mean of n)×100]. A %CV of ≤20% was considered an acceptable result.

3.3 Results

3.3.1 Specificity

Depletion studies on pooled wound fluids showed that TR-FIA1, TR-FIA2 and TR-FIA3 had cross-reactivities of 3%, 1% and 20% respectively (Table 3); while the same assays had cross reactivities of 42%, 25% and 23% when depleted tissue homogenates were analysed (Table 4).

Table 3: Specificity analysis by depletion of suPAR from pooled wound fluids. SEM=Standard error of mean.

	Non-depleted wound exudates (ng/ml)±SEM	Depleted wound exudates (ng/ml)±SEM	Cross-reactivity
TRFIA1 (suPAR I-III)	19.5 ± 1.48	0.77 ± 0.11	3%
TRFIA2 (suPAR I-III, suPAR II-III)	114.6 ± 5.21	1.97 ± 0.07	1%
TRFIA3 (suPAR I)	20.29 ± 0.86	4.2 ± 0.04	20%

Table 4: Specificity analysis by depletion of suPAR from tissue homogenates.

SEM=Standard error of mean.

	Non-depleted tissue homogenates (ng/ml)±SEM	Depleted tissue homogenates (ng/ml)±SEM	Crossreactivity
TRFIA1 **(suPAR I-III)**	7.73 ± 2.15	3.31 ± 0.59	42%
TRFIA2 **(suPARI-III, suPAR II-III)**	11.16 ± 3.07	2.87 ± 1.03	25%
TRFIA3 **(suPAR I)**	7.49 ± 0.58	1.73 ± 0.17	23%

3.3.2 Linearity

Serial dilutions of pooled wound fluids ran parallel to the standard curves in all three assays (Fig 7-9). Diluted samples for tissue homogenates remained parallel to the standard curve for TR-FIAs 2 and 3. In TR-FIA1 the parallel relationship between the standard curve and diluted tissue homogenate was lost after a dilution of 1/80.

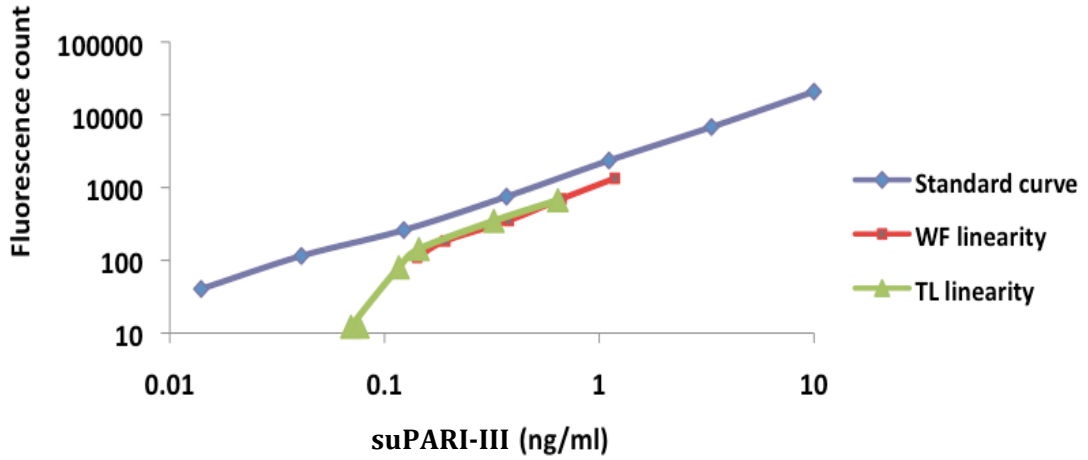


Fig 7. Linearity of sample dilutions TR-FIA1. Pooled wound fluids (WF) and pooled tissue lysates (TL) were serially diluted to 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320. Parallel relationship of tissue lysates was lost after dilution of 1/80, whereas wound fluids maintained a parallel relationship with the standard curve.

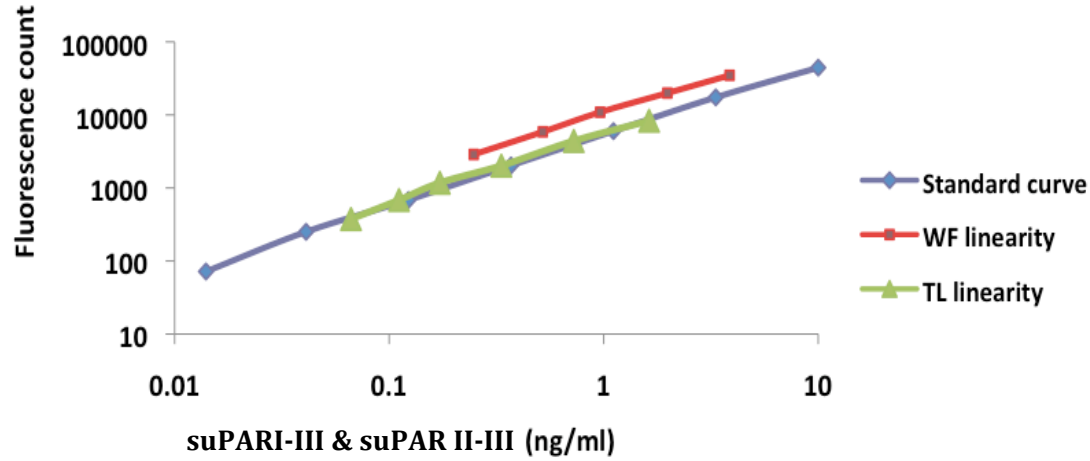


Fig 8. Linearity of sample dilutions in the TR-FIA2. Pooled wound fluids (WF) and pooled tissue lysates (TL) were serially diluted to 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320. Both tissue lystaes and wound fluids maintained a parallel relationship to the standard curve.

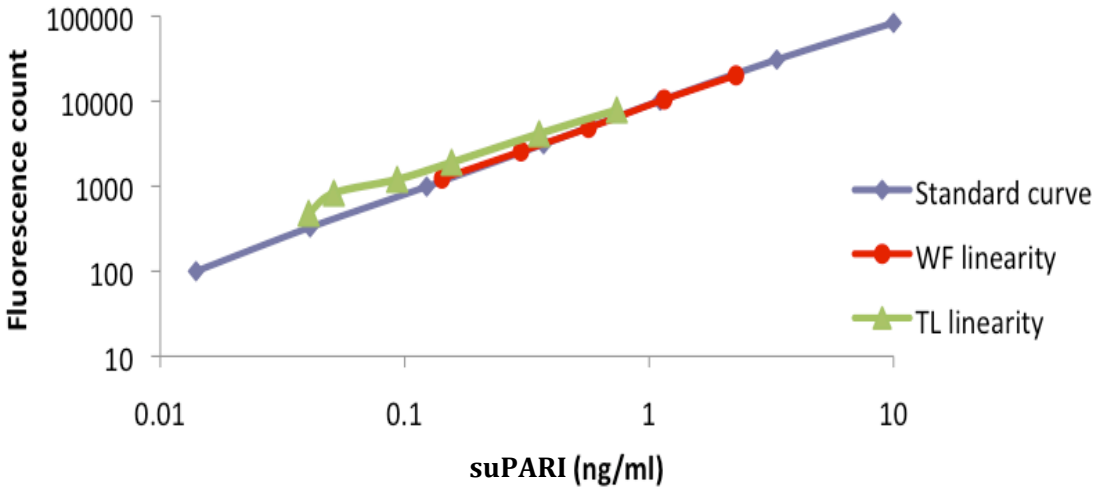


Fig 9. Linearity of sample dilutions in the TR-FIA3. Pooled wound fluids (WF) and pooled tissue lysates (TL) were serially diluted to 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320. Parallel relationship of tissue lysates was lost after 1/80 dilution whereas the wound fluids maintained the parallel relationship with the standard curve.

3.3.3 Accuracy by spike recovery

Recoveries of spiked pooled wound fluids were within 20% of expected concentration for all three assays (Tables 5-7). Recoveries of spiked tissue homogenates remained within 20% of expected concentration in TR-FIA1 and TR-FIA3 (Tables 8-10).

Table 5. TR-FIA1 (suPAR I-III) – Wound fluid spiking

WF pool concentration (ng/ml)	Spike concentration (ng/ml)	Measured WF + spike concentration (ng/ml)	Expected WF + spike concentration (ng/ml)	Difference (ng/ml)	% Recovery
0.02	2	2.03	2.02	0.04	100
0.02	0.4	0.35	0.42	0.07	83
0.02	0.08	0.087	0.1	0.01	87

Table 6. TR-FIA2 (suPARI-III, suPARII-III) – Wound fluid spiking

WF pool concentration (ng/ml)	Spike concentration (ng/ml)	Measured WF + spike concentration (ng/ml)	Expected WF + spike concentration (ng/ml)	Difference (ng/ml)	% Recovery
0.04	2	2.06	2.04	0.02	100
0.04	0.4	0.46	0.44	0.04	104
0.04	0.08	0.12	0.12	0	100

Table 7. TR-FIA3 (suPARI)– Wound fluid spiking

WF pool concentration (ng/ml)	Spike concentration (ng/ml)	Measured WF + spike concentration (ng/ml)	Expected WF + spike concentration (ng/ml)	Difference (ng/ml)	% Recovery
0.02	2	2.06	2.02	0.04	101
0.02	0.4	0.42	0.42	0	100
0.02	0.08	0.08	0.1	0.02	80

Table 8. TR-FIA1 (suPARI-III) - Tissue homogenate spiking

TL pool concentration (ng/ml)	Spike concentration (ng/ml)	Measured TL + spike concentration (ng/ml)	Expected TL + spike concentration (ng/ml)	Difference (ng/ml)	% Recovery
0.02	2	1.99	2.02	0.03	98
0.02	0.4	0.24	0.42	0.18	57
0.02	0.08	0.08	0.1	0.02	80

Table 9. TR-FIA2 (suPARI-III, suPARII-III)- Tissue homogenate spiking

TL pool concentration (ng/ml)	Spike concentration (ng/ml)	Measured TL + spike concentration (ng/ml)	Expected TL + spike concentration (ng/ml)	Difference (ng/ml)	% Recovery
0.1	10	5.9	10.1	4.2	58
0.1	2	0.34	2.1	1.76	16
0.1	0.4	0.11	0.5	0.39	22

Table 10. TR-FIA3 (suPARI) tissue homogenate spiking

TL pool concentration (ng/ml)	Spike concentration (ng/ml)	Measured TL + spike concentration (ng/ml)	Expected TL + spike concentration (ng/ml)	Difference (ng/ml)	% Recovery
0.03	2	2.29	2.03	0.26	112
0.03	0.4	0.43	0.43	0	100
0.03	0.08	0.08	0.11	0.03	72

3.3.4 Intra-assay precision

The intra-assay-variability for the all TR-FIAs in pooled wound fluids was <5% (Table 11). The CVs were always <6% except for TR-FIA1 assay of tissue homogenates (Table 12).

Table 11. Intra-assay precision for wound exudates. Std Dev=Standard deviation. CV=Co-efficient of variation

	Mean (ng/ml)	Std Dev	%CV	n
TR-FIA1 (suPARI-III)	20.90	0.88	4.25	10
TR-FIA2 (suPARI-III, suPARII-III)	43.33	1.09	2.51	10
TR-FIA3 (suPARI)	20.42	0.72	3.52	10

Table 12. Intra-assay precision for tissue homogenates. Std Dev=Standard deviation. CV=Co-efficient of variation

	Mean (ng/ml)	Std Dev	%CV	n
TR-FIA1 (suPARI-III)	7.18	1.96	27.31	10
TR-FIA2 (suPARI-III, suPARII-III)	8.65	0.48	5.56	10
TR-FIA3 (suPARI)	6.72	0.53	3.52	10

3.4 Discussion

The results of thse experiments show that the TR-FIA 1, 2 and 3 can be used to measure suPAR and its fragments in wound exudates. The generally poor validation results for tissue homogenates (selectivity, linearity and precision) may have been the result of the high level of interference by substances or matrix revealed by the depletion studies, even though linearity on the whole was reasonable. These assays were therefore considered not fit for use in quantifing suPAR forms in tissue homogenates and were therefore only used to quantify suPAR forms in wound exudates in the subsequent experiments in this thesis.

The plasminogen system consists of many components including uPA, uPAR and the inhibitors PAI-1 and PAI-2, and therfeore an ideal extraction solution (buffer) should be capable of extracting all the components of the plasminogen system. Some buffers give a higher extraction efficiency of PAI-1 at the expense of uPA and uPAR, whereas others are potent extractors of uPAR only[181]. One solution to this problem is to use different extraction buffers on the same sample in order to obtain the maximum amount of uPAR, uPA and PAI. This was not possible in this study as the tissue amounts of ulcer tissue available from the 6 mm punch biopsy were too small to carry out all the possible extractions. We therefore used an extraction solution that optimally extracted, uPAR [181] as this was the main focus of this project.

Chapter 4: Quantification of suPAR and its fragments in wound exudates and plasma

4.1 Introduction

The amount of suPAR and its fragments (suPAR I & suPAR II-III) was measured in venous ulcer exudates, acute wound exudates and blood plasma using TR-FIAs. The aim was to compare the difference in expression of these fragments in healing and non-healing venous ulcers. Relationship between systemic and local expression of suPAR fragments was also analysed

4.2 Methods

The methods used were similar to the ones used for validation of suPAR. Each wound exudate sample was diluted to 1/15 in delfia assay buffer (PerkinElmer). Levels of suPAR and its fragments were adjusted according to the total soluble protein content of each sample. Levels of suPAR and its fragments obtained from venous ulcer and plasma of same patients were also compared to assess any relationship between systemic and local expression of suPAR and its fragments.

Levels of suPAR fragments were calculated in both nanogram per milliliter and nanogram per milligram of soluble protein. There is no standard method used for measurement of micromoleclues within wound fluids. In previous studies

proteins and cytokines within wound fluids have been measured both in relationship to volume of fluid[182] as well as soluble protein content[95]. The main reason to standardize measurement of samples according to soluble protein content of the sample is to nullify the effect of hydration, heart failure and bacterial contamination[183]. We have therefore utilized both methods.

4.3 Results

4.3.1 suPAR I-III in wound exudates

When samples were standardized to mg of solule protein healers and acute wound fluid (AWF) had significantly higher levels of complete suPARI-III compared to non-healers. The levels of suPARI-III between AWF and healers were similar with no statistically significant difference (Table 13, Fig 10). Measurements in mg/ml showed significantly higher level of suPARI-III in AWF compared to healers that had a significantly higher level than non-healers (Table 14, Fig 11). Statistical tests were performed using unpaired t test and a P value of <0.5 was considered significant.

Table 13. Comparison of suPARI-III levels (nanogram per milligram of soluble protein) between AWF, healing and non-healing ulcer exudates. Statistical analysis using unpaired t test. SEM= Standard error of mean. NS= Not significant

	Number (n)	Mean suPARI-III (ng/mg sol prot)	SEM	P value
AWF	7	0.60	0.14	NS, 0.005, 0.0005
Healers	9	0.58	0.17	
Non-Healers	21	0.20	0.03	

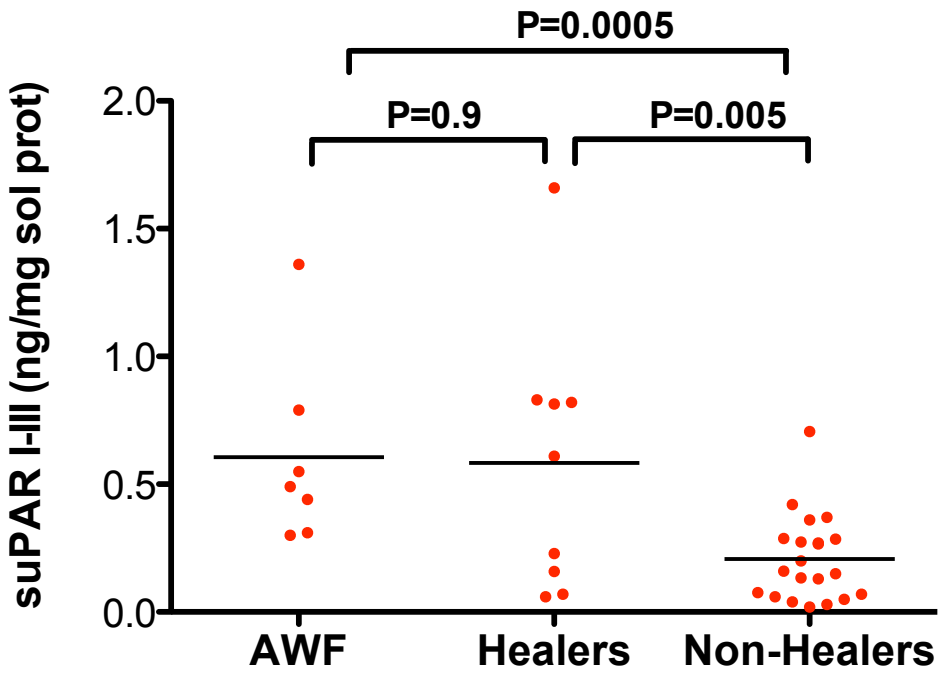


Figure 10: Comparison of suPARI-III levels (ng per milligram of soluble protein) between AWF (acute wound fluids), healing and non-healing ulcer exudates

Table 14: Comparison of suPARI-III levels (nanogram per milliliter) in AWF (acute wound fluids), healing and non-healing ulcer exudates. Statistical test using unpaired t test. SEM= standard error of mean

	Number (n)	Mean suPARI-III (ng/ml)	SEM	P value
AWF	7	26.71	3.39	0.04
Healers	9	14.67	4.2	0.04
Non-Healers	21	7.42	1.37	0.0003

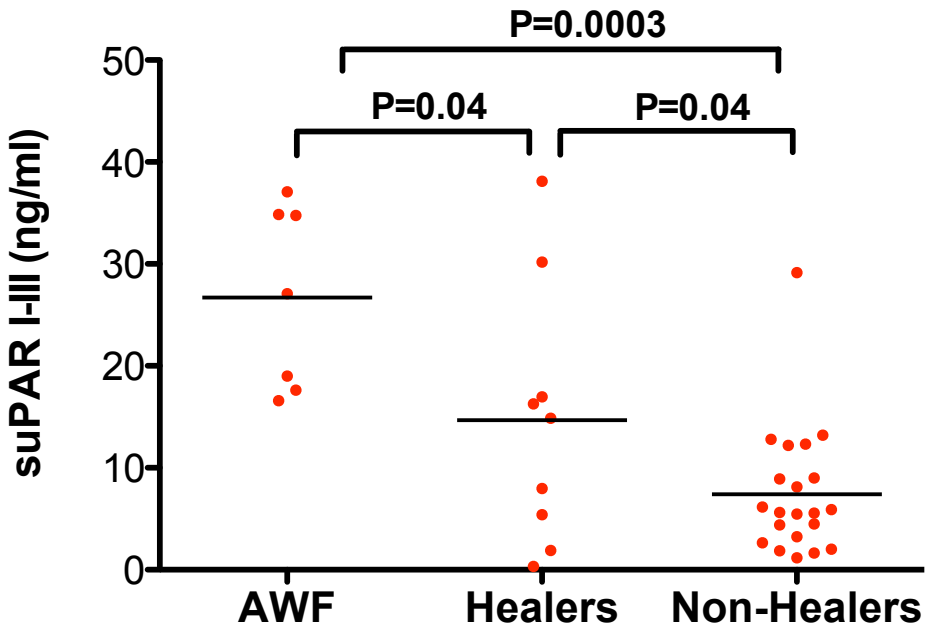


Figure 11: Comparison of suPARI-III levels (nanogram per milliliter) in AWF (acute wound fluids), healing and non-healing ulcer exudates

4.3.2 suPARI in wound exudates

Measurements in ng/mg of soluble protein showed higher levels of suPARI in healers compared to non-healers (Table 15, Fig 12). Comparison of AWF with healers and non-healers did not show any statistically significant difference. Statistical tests for samples measured in ng/mg of soluble protein were performed using unpaired t test as the data was normally distributed. Measurements in ng/ml did not show any significant difference between the samples (Table 16, Fig 13). Statistical tests for samples measured in ng/ml were performed using Mann Whitney test as data was not normally distributed. P value of <0.5 was considered significant

Table 15. Comparison of suPARI levels (nanogram per milligram of soluble protein) between AWF (acute wound fluids), healing and non-healing wound exudates. Statistical tests using unpaired t test. SEM= Standard error of mean

	Number (n)	Mean suPARI (ng/mg sol prot)	SEM	P value
AWF	7	1.89	1.06	NS
Healers	9	1.49	0.42	0.03
Non-Healers	21	0.72	0.13	NS

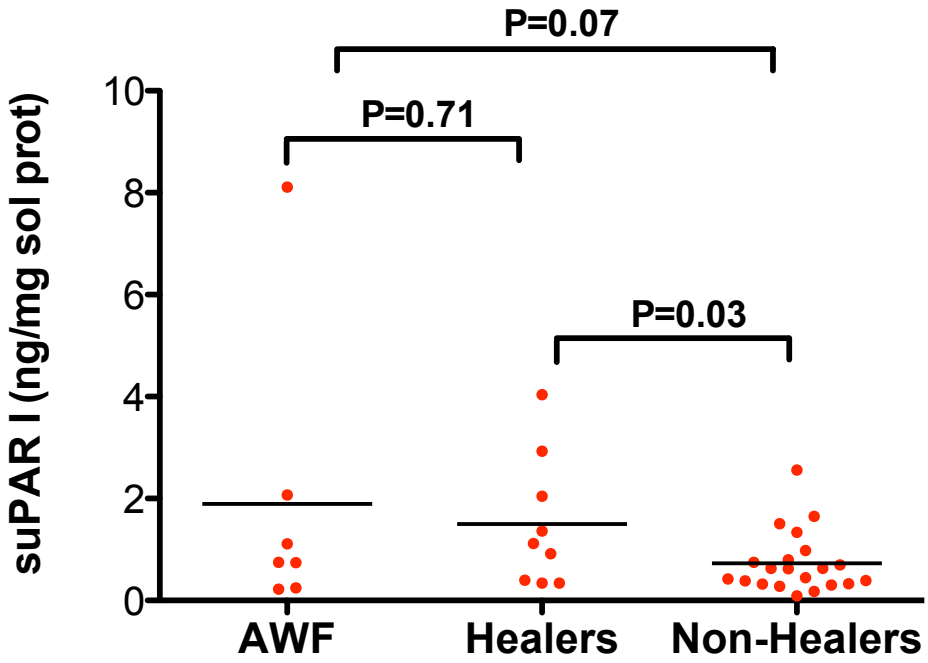


Figure 12: Comparison of suPARI levels (nanogram per milligram of soluble protein) between AWF (acute wound fluids), healing and non-healing ulcer exudates

Table 16: Comparison of suPARI levels (nanogram per milliliter) between AWF (acute wound fluids), healing and non-healing ulcer exudates. Statistical test using Mann Whitney test. SEM=standard error of mean. NS=not significant

	Number (n)	Mean suPARI (ng/ml)	SEM	P value
AWF	7	69.73	29.52	NS, NS, NS
Healers	9	37.84	7.6	
Non-Healers	21	29.38	5.9	

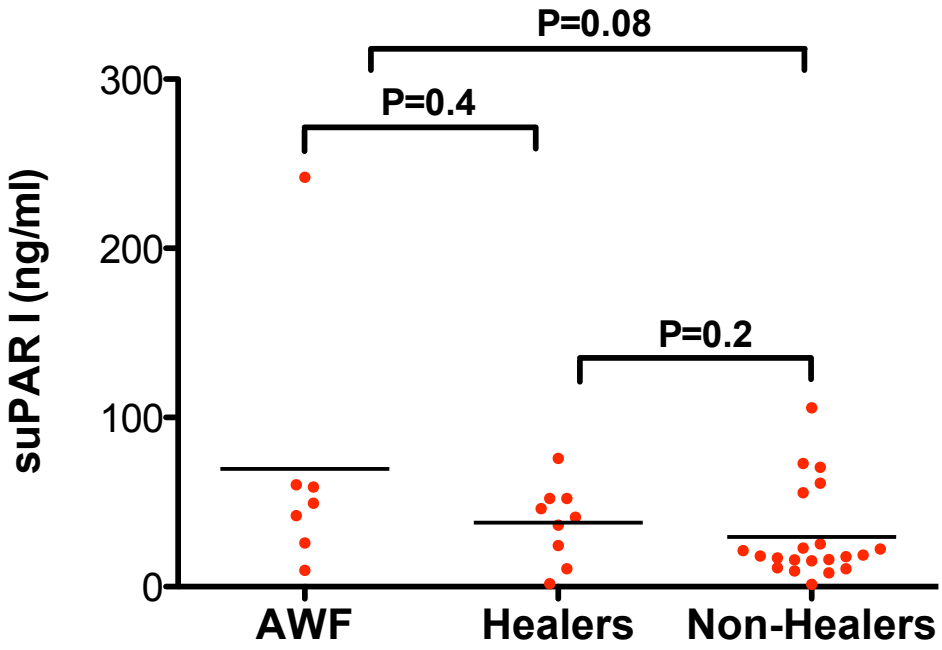


Figure 13: Comparison of suPARI (nanogram per milliliter) between AWF (acute wound fluids), healing and non-healing ulcer exudates.

4.3.3 suPARII-III in wound exudates

Measurements in ng/mg of soluble protein showed that healers had significantly higher levels of suPARII-III compared with non-healers (Table 17, Fig 14). Healers even had a significantly higher level of suPARII-III compared to AWF. There was no difference in suPARII-III between AWF and non-healers. When samples were compared in ng/ml the only significant difference was that healers still had a higher level of suPARII-III compared to non-healers (Table 18, Fig 15). Statistical analyis was performed using unpaired t test and a P value of <0.5 was considered significant.

Table 17. Comparison of uPARII-III levels (nanogram per milligram of soluble protein) in AWF (acute wound fluids), healing and non-healing ulcer exudates. Statistical analysis using unpaired t test. SEM= standard error of mean. NS=not significant

	Number (n)	Mean suPARII-III (ng/mg sol prot)	SEM	P value
AWF	7	1.28	0.59	0.003
Healers	9	4.24	0.58	<0.0001
Non-Healers	21	1.55	0.19	NS

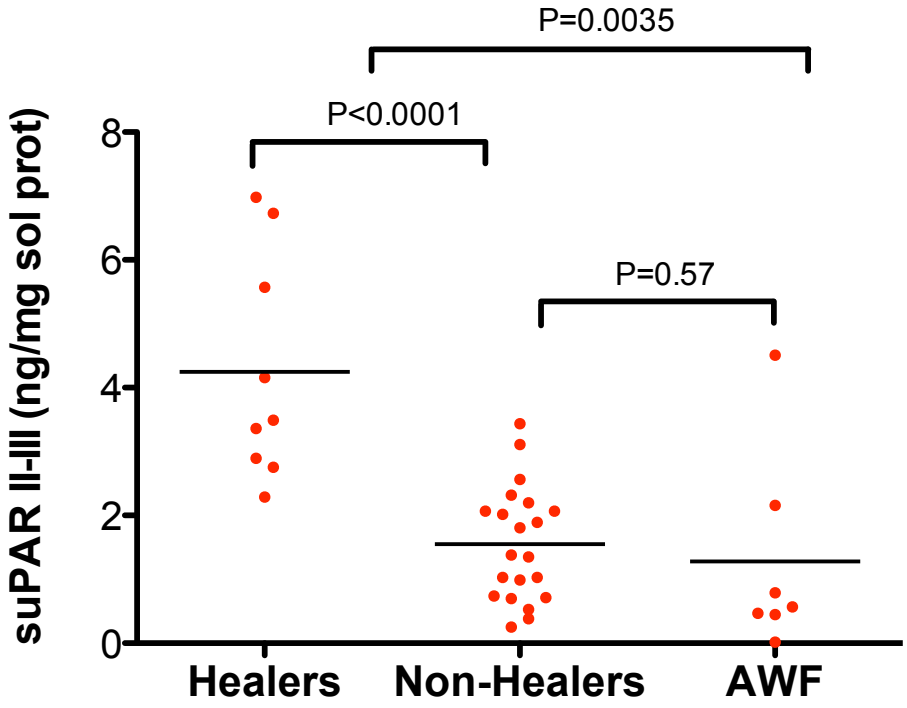


Figure 14: Comparison of suPARII-III levels (nanogram per milligram of soluble protein) between AWF (acute wound fluids), healing and non-healing ulcer exudates

Table 18: Comparison of suPARII-III levels (nanogram per milliliter) between AWF (acute wound fluids), healing and non-healing ulcer exudates. Statistical analysis using unpaired t test. SEM=standard error of mean. NS=not significant

	Number (n)	Mean suPARII-III (ng/ml)	SEM	P value
AWF	7	51.44	16.61	NS, 0.03, NS
Healers	9	121.1	26.79	
Non-Healers	21	65.82	11.07	

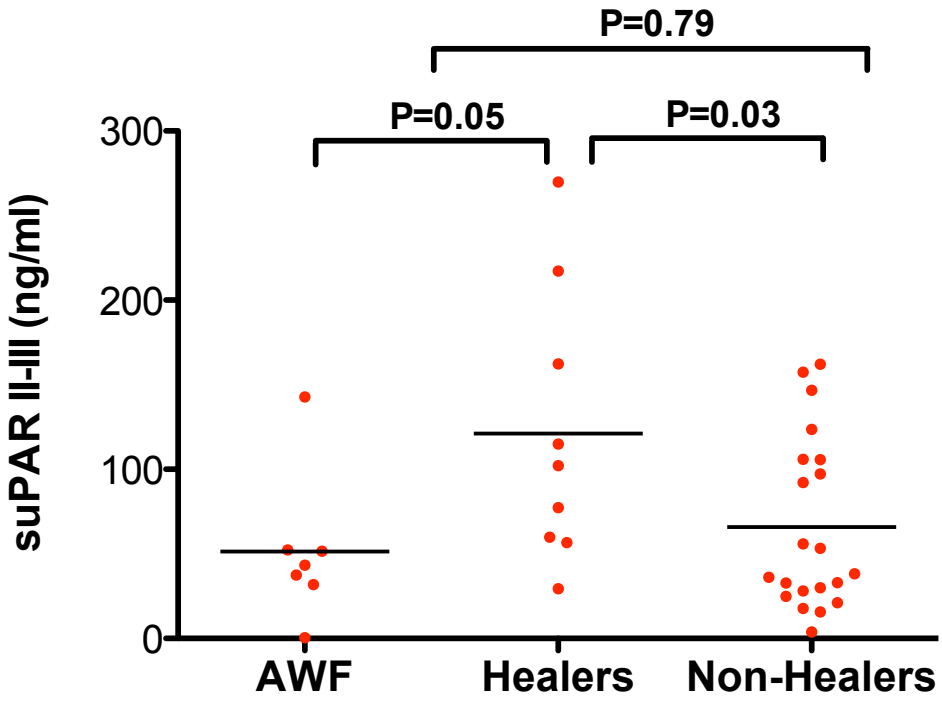


Figure 15: Comparison of suPARII-III levels (nanogram per milliliter) between AWF (acute wond fluids), healing and non-healing ulcer exudates

4.3.4 Plasma suPAR levels

Blood plasma samples (n=10) were obtained from patients at start of compression therapy (7 from non-healing ulcers and 3 from healing ulcers). Although there were only 3 healing ulcers using statistical analysis there appered to be little difference between levels of suPAR and its fragments in healing and non-healing ulcers (Table 19, Fig 16-18). Of note, levels of all suPAR fragments appeared to be lower in plasma compared to the levels in wound exudates of same patients (Plasma suPARI 0.24±0.02ng/ml vs exudate suPARI 42.11±10.04ng/ml; Plasma suPARII-III 1.5±0.19ng/ml vs exudate suPARII-III 118.3±21.53ng/ml; Plasma suPARI-III 1.72±0.63ng/ml vs exudate suPARI-III 15.01±4.04ng/ml).

Table 19: Comparison of plasma suPAR fragments (nanogram per milliliter) between patients who had healing and non-healing leg ulcers. SEM= standard error of mean. NS= not significant

	H (Mean±SEM) n=3	NH (Mean±SEM) n=7	P value
suPAR I (ng/ml)	0.24±0.02	0.24±0.03	NS
suPAR II-III (ng/ml)	1.36±0.48	1.55±0.20	NS
suPAR I-III (ng/ml)	1.42±0.33	1.85±0.91	NS

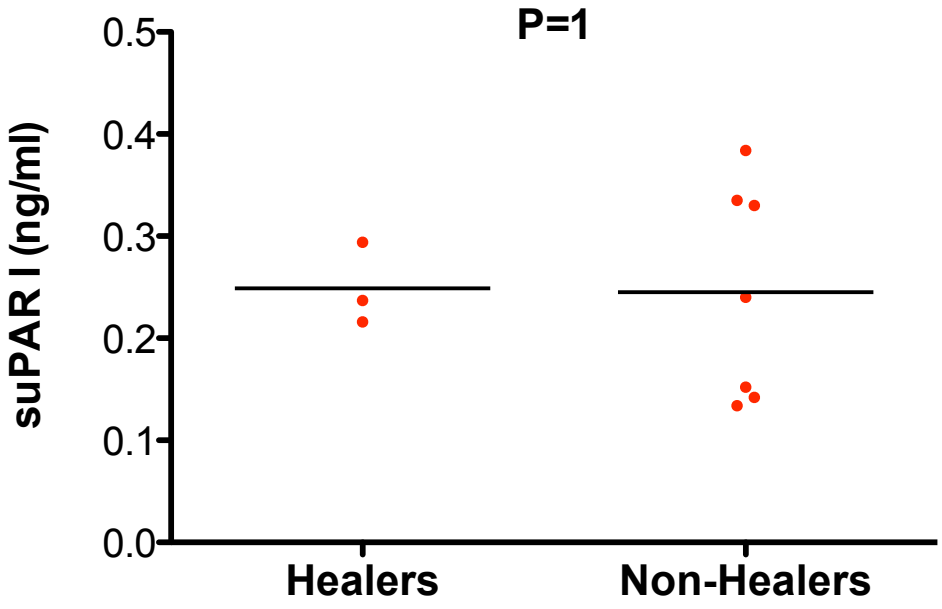


Fig 16. suPARI levels (nagnogram per milliliter) in plasma of healers and non-healers.

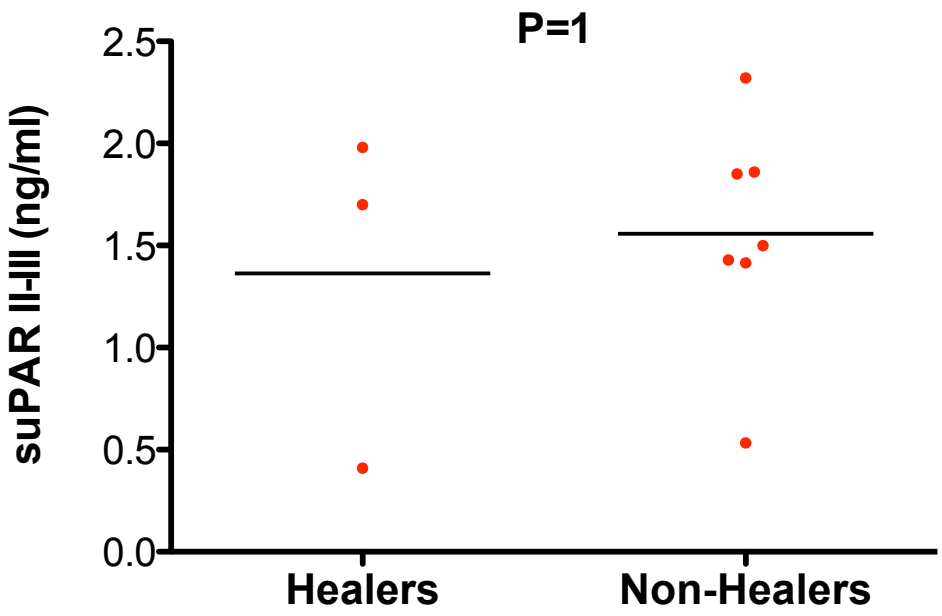


Fig 17. suPARII-III levels (nanogram per milliliter) in plasma of healers and non-healers.

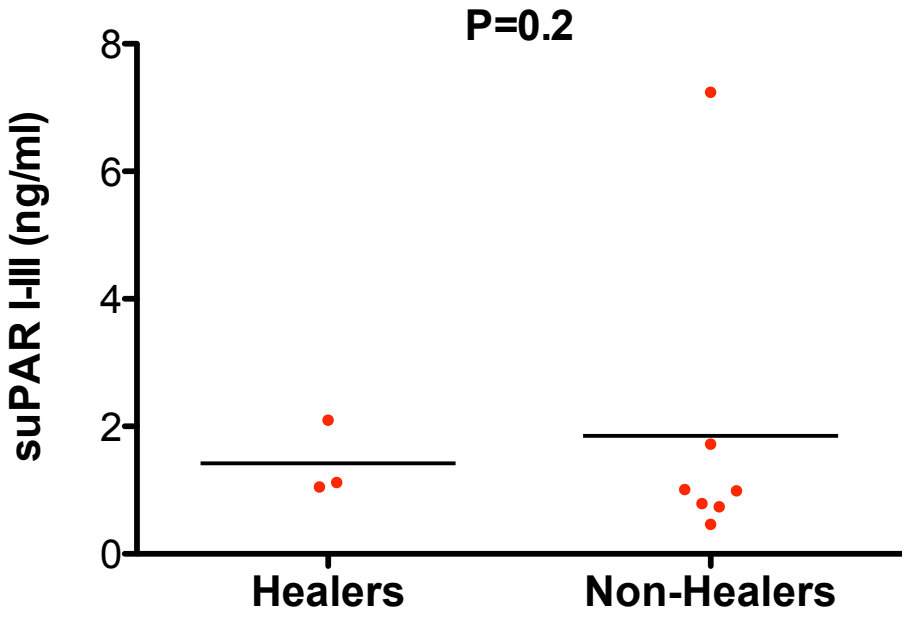


Fig 18. suPARI-III levels (nanogram per milliliter) in plasma of healers and non-healers.

4.3.5 Correlation of between suPAR levels in plasma and ulcer exudates from same patients

There was no correlation between suPAR levels in plasma and ulcer exudates (Fig 19-21). Statistical tests performed using Pearson correlation and a P value of <0.5 was considered significant

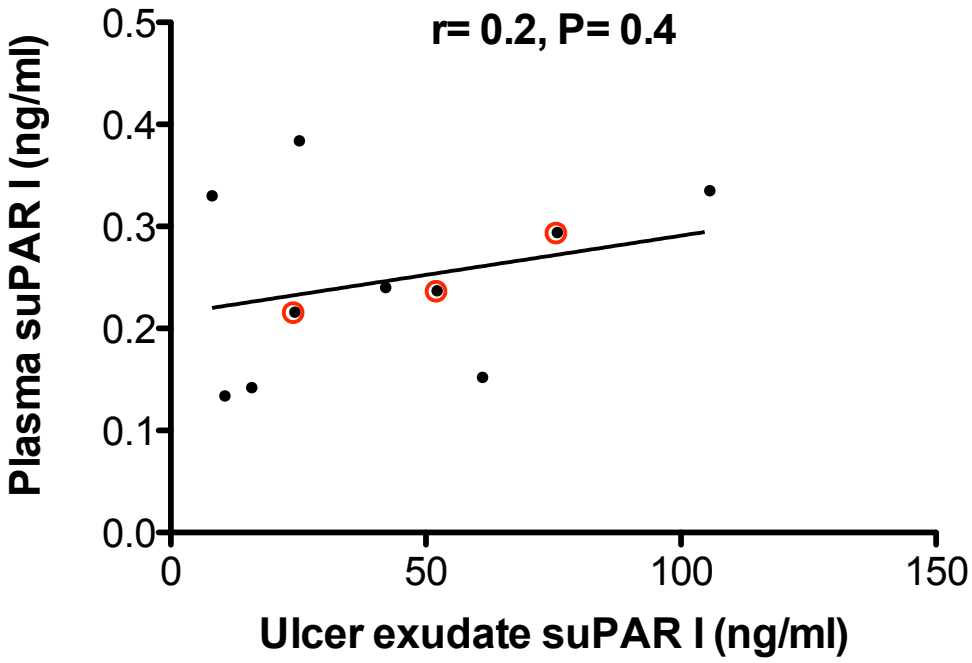


Fig 19. Correlation of suPAR I levels (nanogram per milliliter) between blood plasma and ulcer exudates. Points in red circles represent healers. Statistical analysis by Pearson correlation.

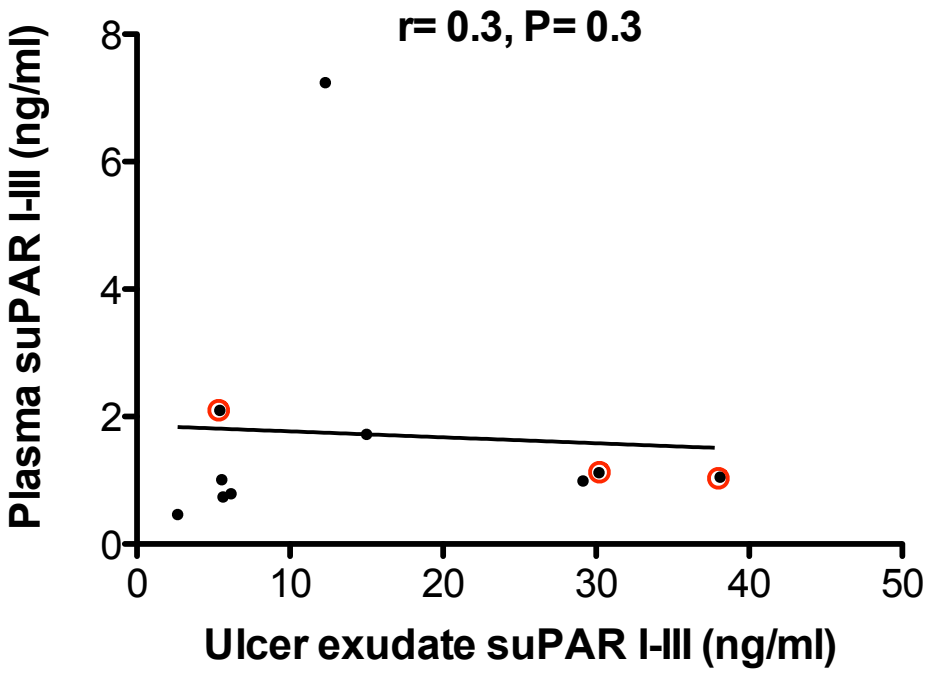


Fig 20. Correlation suPAR I-III levels (nanogram per milliliter) between blood plasma and ulcer exudates. Points in red circles represent healers. Statistical analysis by Pearson correlation.

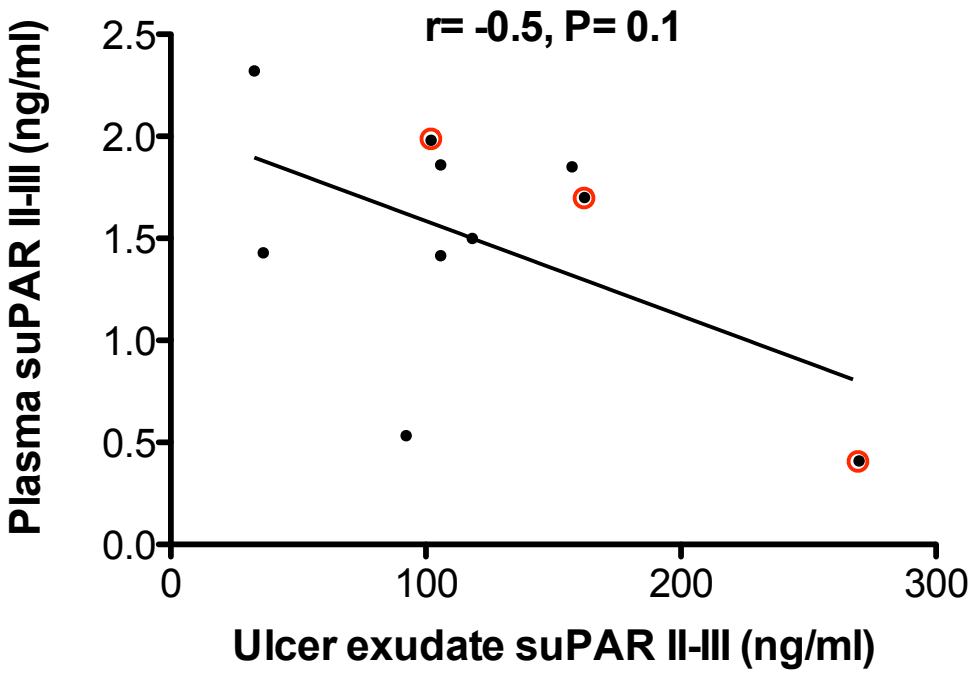


Fig 21. Correlation of suPAR II-III levels (nagnogram per milliliter) between blood plasma and ulcer exudates. Points in red circles represent healers. Statistical analysis by Pearson correlation

4.4 Discussion

There was significantly higher levels of all forms of suPAR in healing ulcer exudates compared with non-healing ulcer exudates suggesting a role for this receptor in ulcer healing. Whether samples were measured in ng/ml or ng/mg of soluble protein suPARI-III and suPARII-III levels were significantly higher in healing ulcer exudates compared to non-healing ulcer exudates. Comparisons with AWF were also made to see if the environs of the healing venous ulcer bore any resemblance to that of an acute wound with respect to suPAR levels. The results obtained show that AWF has lower levels of suPAR II-III compared with

exudates from healing ulcers (and to a lesser extent exudates from non-healers). The greater amounts of this fragment found in ulcer exudates as a whole supports the possibility of a role for this peptide in healing chronic wounds.

Soluble urokinase receptor, containing the chemotactic peptide (suPARII-III) that regulates cellular chemotaxis[154, 170] does not have a role in proteolytic activation of the plasminogen system. This fragment appeared to be the best discriminator between healers and non-healers, suggesting that other non-proteolytic activities of uPAR may be involved in ulcer healing.

The urokinase receptor is an extracellular protein that is dependent on trans-cellular proteins like integrins for regulating cellular functions[152], suggesting possibility of interaction with other micromoleclues within the ulcer environment. Animal ulcer models show that cellular processes important for ulcer healing rely on expression of uPAR on cell surface, including keratinocyte proliferation and migration[165], angiogenesis[184] and inflammatory cell infiltration[145]. Murine keratinocytes from uPAR knock-out mice migrate more slowly than keratinocytes from wild type mice[165]. It remains unclear, however as to which of these pathways may be activated in ulcer healing in man. What stimulates increased expression of suPAR in healing ulcers or inhibits its expression in non-healing ulcers, merits further investigation.

Previous studies have suggested release of suPAR into the blood from patients with advanced breast and colon cancers[185] as well as within biological fluids (urine) of healthy volnteers[186]. Ulcer exudates represent a filtrate of plasma together with factors secreted by cells. We wanted to investigate whether the levels in the exudates from chronic venous ulcers would be reflected in blood.

The levels of circulating suPAR and its fragments were orders of magnitude lower than in the exudates, suggesting that there is a substantial contribution to suPAR exudate levels from the local wound tissue. We found no correlation between suPAR levels in exudates and plasma, but these conclusions must be assessed with caution due to the fact that only small sample numbers were available for this analysis.

The restriction to 10 blood samples by the eithcal committee prevented a useful interpretation of the results. After analyzing the plasma samples a substantial amendment was submitted to the eithcal committee for increasing the number of plasma samples. This however was not very useful as it was only done towards the end of the study after the validation experiments. We hope this based on our limited plasma sample results future studies will be given more linency for obtaining blood samples.

Chapter 5: uPA protein, uPA activity and PAI-1 levels in venous ulcer tissue and wound exudates

5.1 Introduction

The amount of total urokinase activator (uPA) is higher in wounds compared with healthy skin [117, 187]. Higher expression in addition to increased bioactivity of uPA is also seen in venous leg ulcers[112]. Other components of plasminogen system e.g. PAI-1 is also present within the environment of venous ulcers, where it is reported increased compared with normal skin[179].

The aim of this section of this thesis was to determine whether there was a difference in the amount and activity of these componets of the plasminogen system in the environs of healing and non-healing venous ulcers.

5.2 Method for measuring total uPA

Total uPA was measured using a commercially available ELISA kit (IMUBIND®; American Diagnostica), which contained a 96-well plate pre-coated with primary antibody. It has been designed to measure all forms of uPA including receptor bound uPA as well as uPA bound to PAI complexes in wound exudates and tissue homogenates (American Diagnostica).

Statistical analysis between the groups was performed by using Mann Whitney test and a P value of <0.05 was considered significant. As described previously

all wound exudate samples were calculated in both ng/mg of soluble protein and ng/ml

5.2.1 Reagents

Standards were prepared by adding 1ml of deiodinized water to the provided standards with concentration ranging between 0.10 and 1ng/ml, were used.

Detection Antibody: prepared by adding 5.5ml of deiodinized water to the powdered form and mixed gently for 3 mins.

Enzyme conjugate diluent: prepared by adding 20 ml of deiodinized water to the provided vial and mixing it.

Wash buffer: prepared by adding 4ml of 25% Triton X-100 to 996 ml of PBS.

Substrate (Tetramehtylbenzidine): provided with the kit.

Stop solution: $2MH_2SO_4$

5.2.2 Assay steps

1. Duplicate wells were filled with 100µl of standard or diluted samples.
2. Plate was covered with a plastic seal and incubated overnight at 4°C.
3. The next day plate was washed with wash buffer 4-times.
4. 100µl of detection antibody was added to each well. The plate was covered with a plastic seal and incubated at room temperature for 1hr.
5. Plate was washed again 4-times, with the wash buffer.

6. For a 96-well plate 12µl of enzyme conjugate was added to 12ml of enzyme conjugate diluent. 100µl of the diluted enzyme conjugate was added to each well, followed by sealing and incubating the plate for one hour.
7. Plate was washed again 4-times, with the wash buffer.
8. 100µl of substrate solution was added to each well. The plate was covered and incubated for 20mins while protecting it from light.
9. The reaction was stopped by adding 50µl of stop solution per well.
10. The absorbance of each well was measured using in a microplate reader (Spectromax, Moledular Dynamics) at a wavelength of 450nm.

5.3 Method for measuring active uPA

UPA activity was measured by bioimmunoassay using the World Health Organization uPA standard (National Institute for Biological Standards and Controls, Hertfordshire, UK) as previously described [177].

Statistical analysis was performed by using Mann Whitney test and a P value of <0.05 was considered significant

5.3.1 Reagents for uPA activity

The following additional materials were used:

1. **Sheep anti uPA antibody:** obtained from Prof Patrick Gaffney (National Institute for Biological Standards and Controls, NIBSC).
2. **Wash buffer**: 0.05% of Tween20 in phosphate buffer saline (PBS).

3. **Blocking buffer**: 1% bovine serum albumin in PBS.
4. **Chromogenic substrate**: S2251 chormogenic substrate (ChromogeniX) (2ml) and 40μl of lys plasminogen diluted in 8ml of wash buffer.

5.3.2 Assay steps

1. Sheep anti uPA antibody was coated on a 96 well plate 100μl per well after diluting 10μl of the antibody in 10ml of Phosphate buffer saline (PBS).
2. The plate was then incubated at 4°C overnight.
3. Next day plate was washed 4 times with wash buffer.
4. Plate was blocked, by adding 200μl of blocking buffer in each well. It was then incubated at room temperature for one hour.
5. After another 3 washes standards and samples were added in duplicates, 100μl per well.
6. The plate was reincubated at 4°C overnight.
7. Next day plate was again washed 3 times with wash buffer
8. 100 μl of S2251 subtsrate was added to each well.
9. Plate was incubated at room temperature while shaking
10. Readings were taken at 405nm at 1, 2 and 3hrs.

Chronic wound exudates, acute wound fluids (n=7) and all tissue homogenates were analysed for the presence of uPA activity. The standards were in the concentration range 0.01-100IU/ml. Chronic wound fluids were diluted to 1/2.5, 1/5, 1/10 and 1/40, and acute wound fluids, 1/5, 1/10, 1/50 and 1/100. Tissue homogenates were diluted to 1/10, 1/50 and 1/100. Correlation

between active uPA within tissue homogenates and total PAI-1 was also analysed.

5.4 Method for measuring PAI-1

Plasminogen activator inhibitor-1 was measured in both wound exudates as well as tissue homogenates using a commercial ELISA kit (R&D DuoSet-DY1786). This ELISA kit detects both latent and active froms of PAI-1 in addition to the PAI-1 bound to vitronectin. Wound exudates and tissue homogenates were collected as previously described. The assay was validated for wound fluids by determining loss of linearity and recovery of spike in a pool of wound fluids. For linearity wound fluid pool was diluted to 1/4, 1/8, 1/16, 1/32 and 1/64. Spiking was carried out with 10ng/ml, 2ng/ml and 0.4ng/ml of PAI-1 standard added to a pooled exudate. Recovery was defined as percentage of difference between measured concentration and actual difference. Percentage spike recovery was calculated using the formula [(Measured sample+spike/Expected sample+spike)×100]. Recovery of 100±20 was considered an acceptable result

Assay steps

1. Assay dilutent (50µl) was added to all wells in the 96-well microtitre plate that was pre-coated with primary mouse monoclonal PAI1 antibody.

2. Standards (20-0.156 ng/ml) and samples (50µl) were added to respective well, followed by incubation at room temperature for 2hrs.

3. Each well was aspirated and washed 4times with wash buffer (400µl/well) using a automated micrititre plate washer.

4. Addition of 200µl of PAI-1 conjugate was followed by incubation of the wells at room temperature for 2hrs

5. Each well was again aspirated and washed 4times followed by addition of 200µl of substrate solution to each well and incubated at room temperature for 30mins in the dark.

6. The reaction was stopped by addition of 50µl of stop solution to each well.

7. The absorbance of the reaction product was measured on a microplate reader at a wavelength of 540nm.

5.5 Results

5.5.1 uPA protein

Wound exudates from 9 healers and 21 non-healers were analysed for total uPA protein. There was no significant difference in the levels of uPA found in exudates from healing and non-healing venous ulcers using both methods of measurement (Table 20,21. Fig 22, 23). Acute wound fluids had a significantly higher level of total uPA when compared with non-healing exudates (Table 20,21).

Table 20: Comparison of total uPA level (nanogram per milligram of soluble protein) between AWF (acute wound fluids), healing and non-healing ulcer exudates. SEM= standard error of mean. NS= not significant

	Number (n)	Mean uPA (ng/ mg sol prot)	SEM	P value
AWF	7	0.85	0.30	NS
Healers	9	0.44	0.17	NS
Non-Healers	21	0.23	0.02	0.02

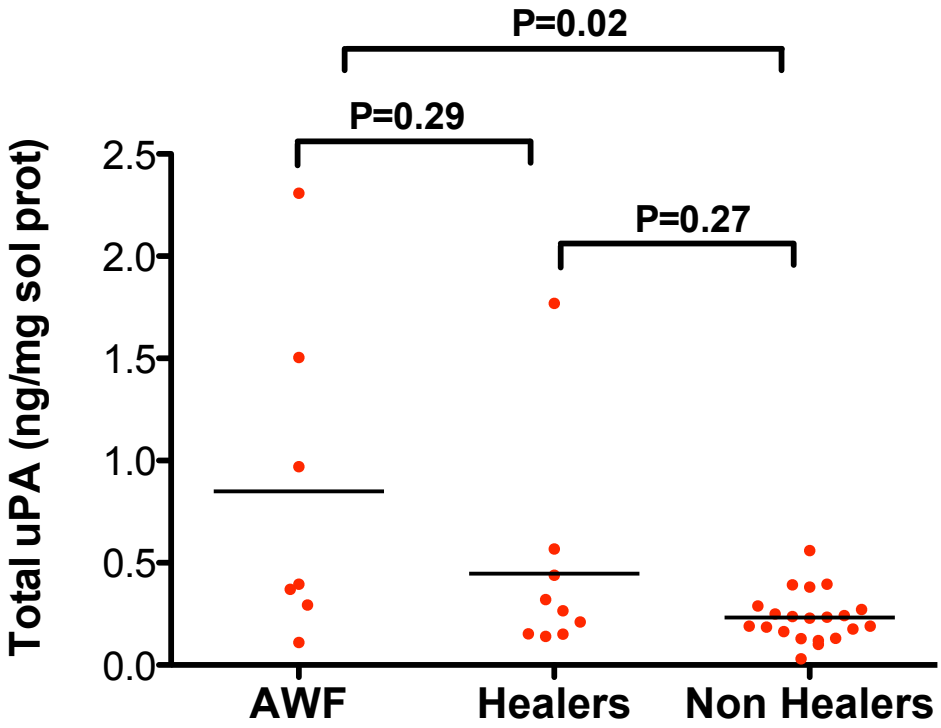


Fig 22: Compaison of total uPA level (nanogram per milligram of soluble protein) between AWF (acute wound fluids), healing and non-healing ulcer exudates. Statistical analysis by Mann-Whitney test

Table 21: Compaison of total uPA level (nanogram per millliter) between AWF (acute wound fluids), healing and non-healing ulcer exudates. SEM= standard error of mean. NS= not significant

	Number (n)	Mean uPA (ng/ ml)	SEM	P value
AWF	7	32.5	6.94	0.001
Healers	9	8.8	0.7	NS
Non-Healers	21	9.1	1.09	0.0006

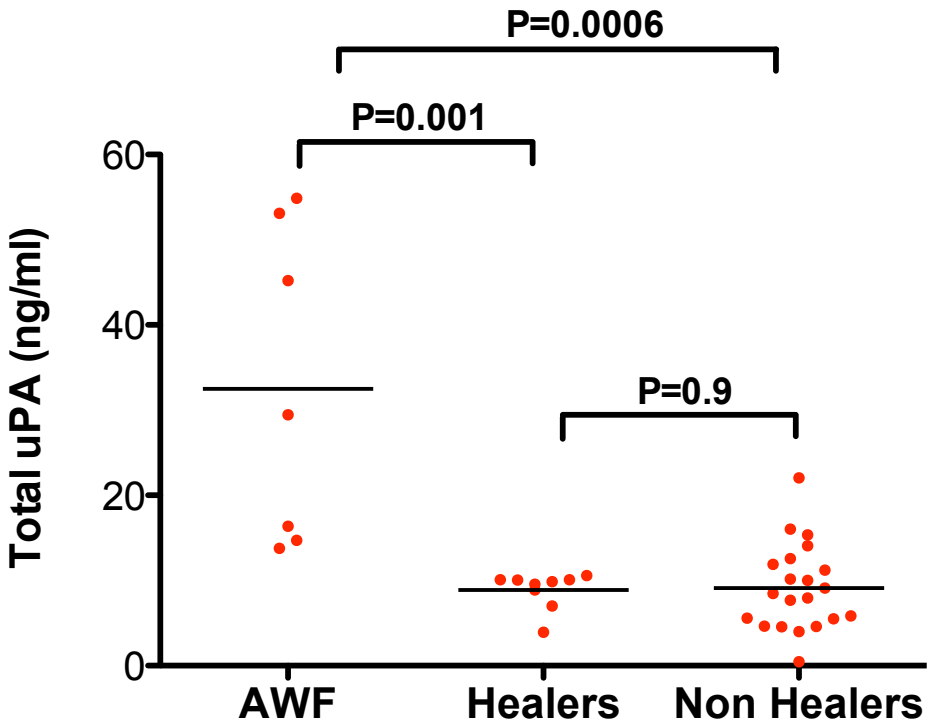


Figure 23: Compaison of total uPA level (nanogram per millliter) between AWF (acute wound fluids), healing and non-healing ulcer exudates. Statistical analysis by Mann-Whitney test

UPA protein was measured in 20 non-healing and 7 healing tissue homogenates. Samples in which uPA levels were below detection level of the ELISA (2 non-healing and 1 healing ulcer homogenate) were given a value 1/10 of the lowest standard. There was no significant difference in total uPA antigen levels in tissue homogenates obtained from healing (mean 2.23ng/mg±1.67) and non-healing (mean 1.41ng/mg±0.97) leg ulcers (P=0.27, Fig 24).

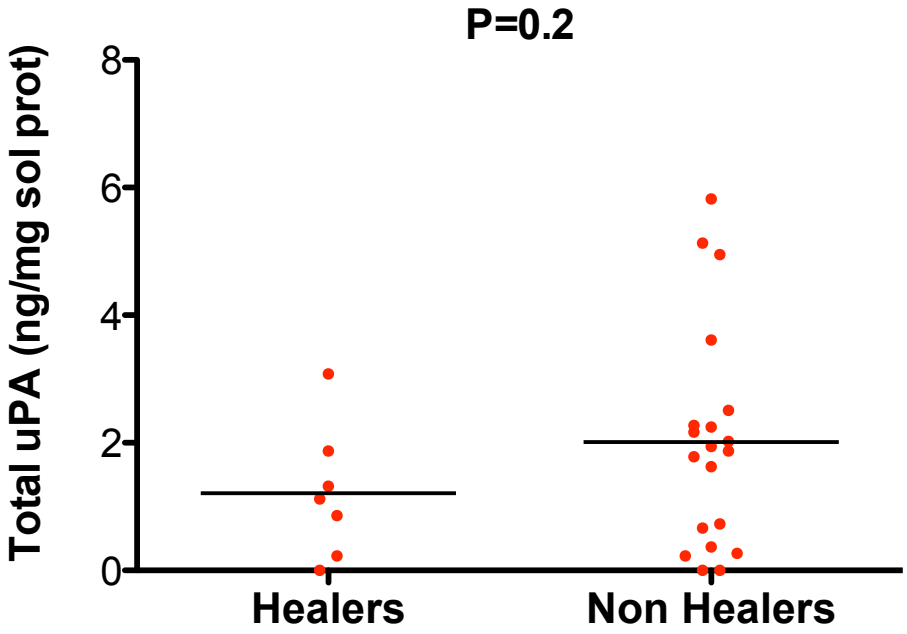


Fig 24: Comparison of total uPA in tissue homogenates obtained from healers and non-healers. Statistical analysis using Mann Whitney test. NS= not significant

5.5.2 uPA activity

There was no detectable uPA activity in any of the wound exudates. Only 11/20 non-healing and 3/7 healing tissue homogenate samples showed a detectable uPA activity. For the purpose of analysis tissue homogenates that had no detectable uPA activity were given a value of 1/10 of the lowest standard. The difference between uPA activity of healing and non-healing tissue homogenates was not statistically significant with both methods of measurements (Fig 25,26).

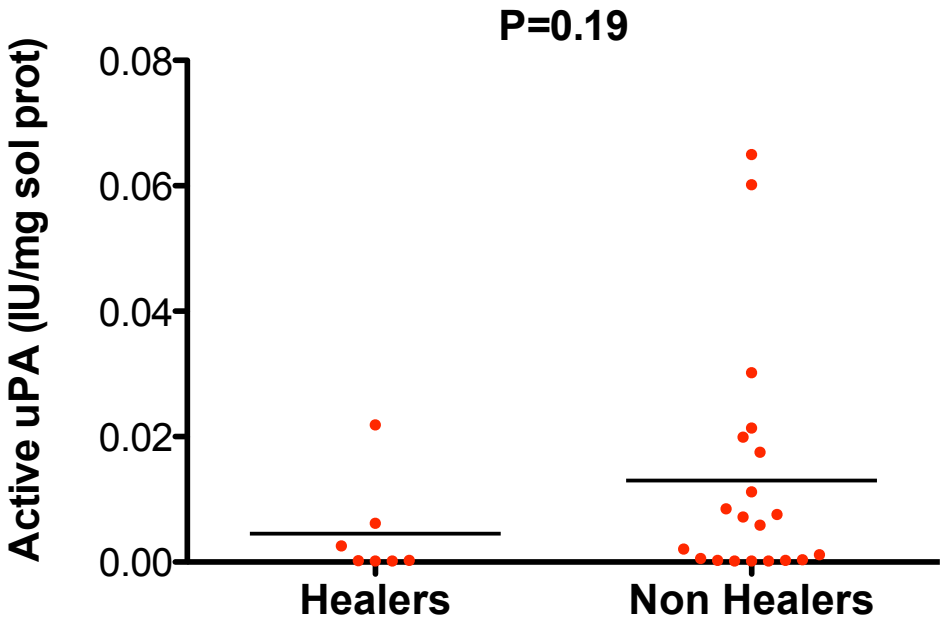


Fig 25: Comparison of uPA activity (milligram per soluble protein) in tissue homogenates from healing and non-healing venous ulcers. Statistical analysis by Mann Whitney test. NS= not significant.

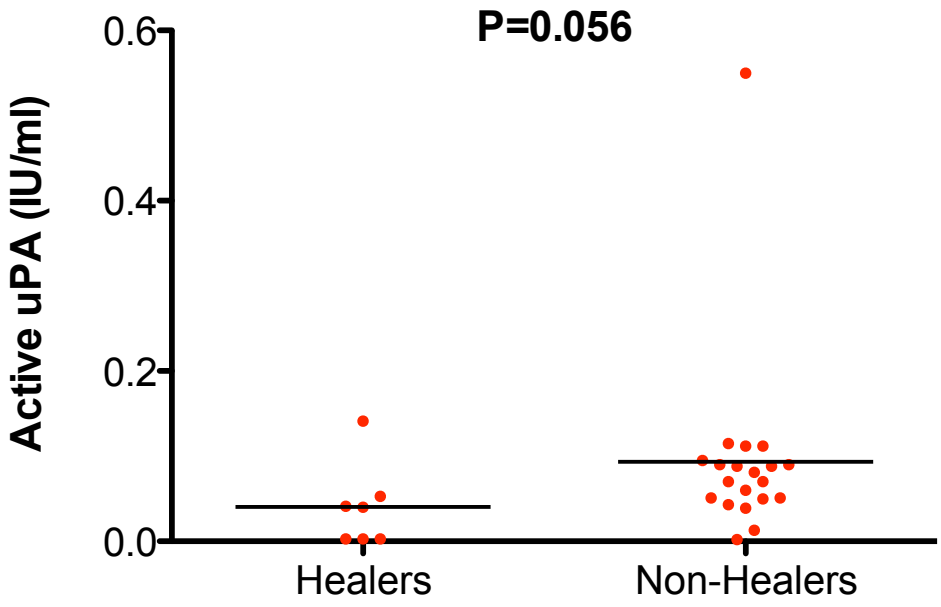


Figure 26: Comparison of active uPA (milligarm per milliliter) in tissue homogenates obtained from healing and non-healing ulcers. Statistical analysis by Mann Whitney test.

5.5.3 Linearity of wound fluid dilution in PAI-1 assay

Serially diluted pool of wound fluid remained parallel to standard curves (Fig 27)

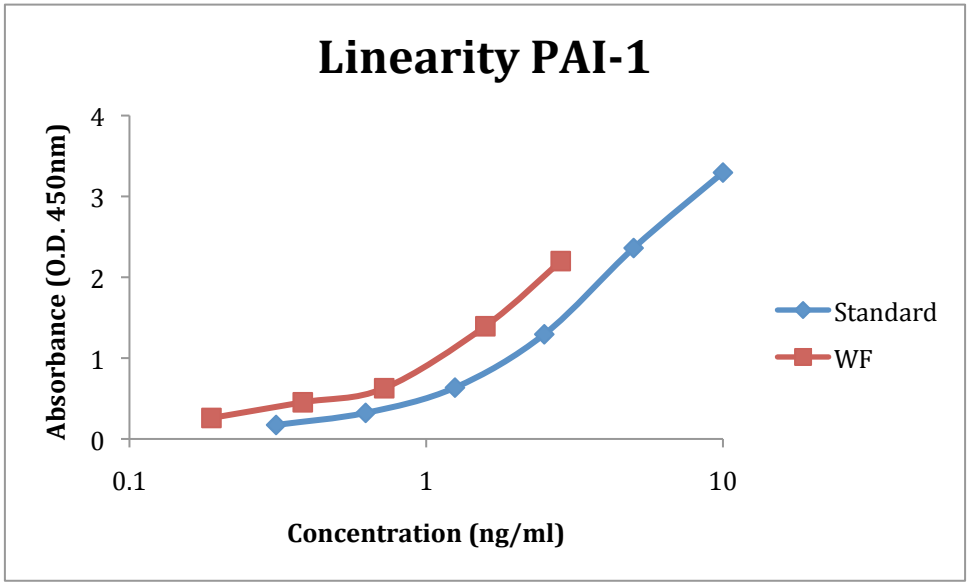


Fig 27: Linearity of wound fluid dilution in the PAI-1 assay

5.5.4 Spiking of wound exudates in the PAI1 assay

Recovery of all the spiked samples was within the acceptable limit of 100±20 % (Table 22)

Table 22: Recovery of wound fluid pool after PAI-1 spiking

WF pool concentration (ng/ml)	Spike concentration (ng/ml)	Measured WE + spike concentration (ng/ml)	Expected WE + spike concentration (ng/ml)	Actual Difference (ng/ml)	% Recovery
0.8	10	11.4	10.8	1.4	105
0.8	2	3	2.8	0.4	107
0.8	0.4	1.08	1.2	0.5	90

5.5.5 Quantification of PAI-1 in exudates and tissue homogenates

When results were calculated according to soluble protein of the sample there was over twice as much PAI-1 in exudates collected from healers than those from with non-healers, but this did not reach significance (P=0.09, Table 23, Fig 28). Acute wound fluids had lowest levels of PAI-1 compared with healing and non-healing exudates (P=0.0007 - healers, P=0.003 – non-healers).

Comparing the samples in ng/ml still showed lowest levels of PAI-1 in AWF but the only statistically significant difference was seen between non-healers and AWF (Table 24, Fig 29)

There were higher levels of PAI-1 in homogenates obtained from non-healing ulcers compared with healing ulcers. The difference was, however, not significant (P=0.09, Fig 30).

Table 23: Comparison of PAI-1 levels (ng per milligram of soluble protein) between AWF (acute wound fluids), healing and non-healing ulcer exudates. SEM= Standard error of mean. NS= not significant

	Number (n)	Mean PAI-1 (ng/ mg sol prot)	SEM	P value
AWF	7	0.12	0.03	0.0007
Healers	9	1.39	0.39	NS
Non-Healers	21	0.69	0.14	0.003

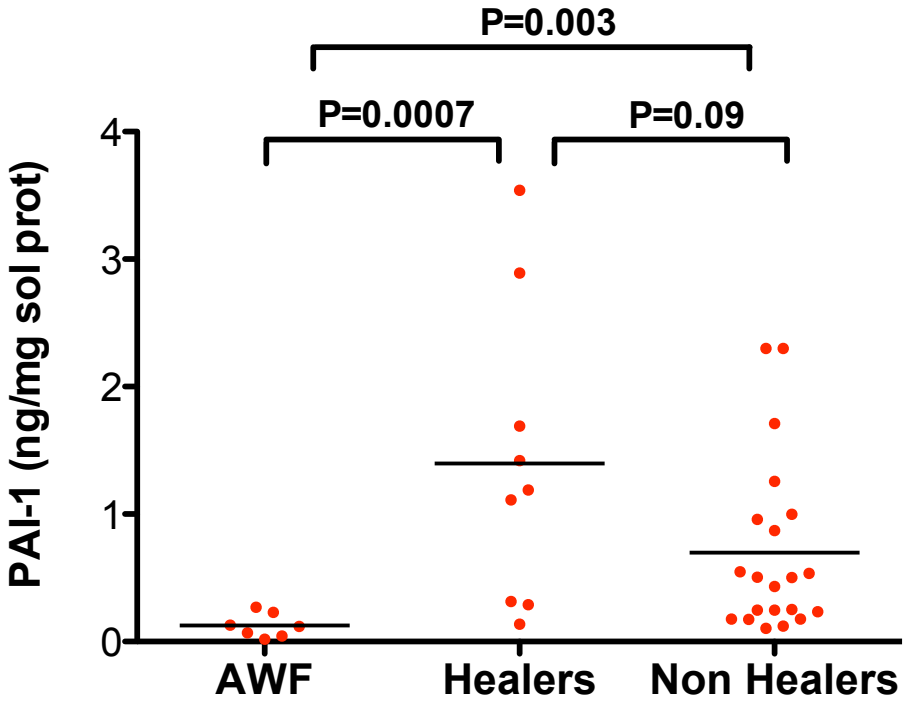


Fig 28: Comparison of PAI-1 levels (nanogram per milligram of soluble protein) betwen AWF (acute wound fluids), healing and non-healing ulcer exudates. Statistical analysis using Mann-Whitney test

Table 24: Comparison of PAI-1 levels (nanogram per milliliter) between AWF (acute wound fluids), healing and non-healing ulcer exudates

	Number (n)	Mean PAI-1 (ng/ ml)	SEM	P value
AWF	7	8.19	1.79	NS, NS, 0.02
Healers	9	29.35	8.25	
Non-Healers	21	24.24	4.72	

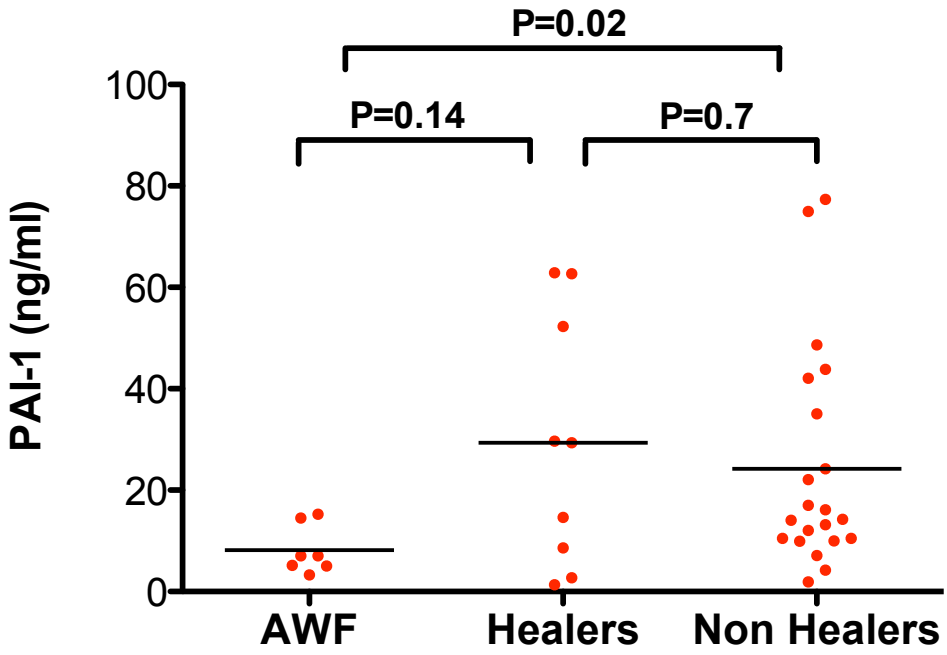


Figure 29: Comparison of PAI-1 levels (nanogram per milliliter) between AWF (acute wound fluids), healing and non-healing ulcer exdates. Statistical analysis using Mann-Whitney test

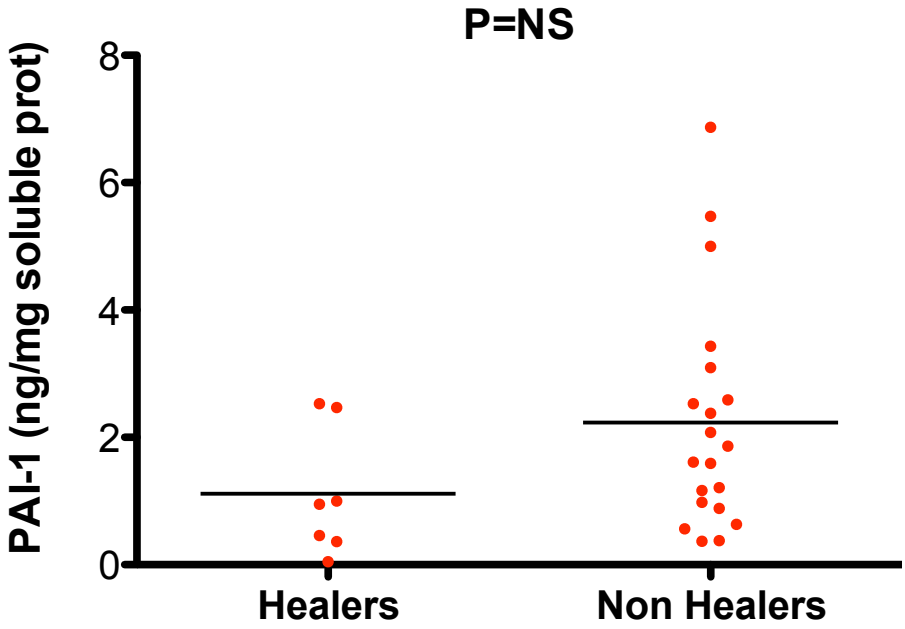


Fig 30: PAI-1 levels in tissue homogenates obtained from healing and non-healing venous ulcers. Statistical analysis using Mann Whitney test. NS=not significant

5.5.6. Correlation between PAI-1 and uPA activity

There was no correlation between active uPA and PAI-1 in the tissue homogenates when analysing data from both healing and non-healing together (Fig 31, P=0.17, Spearman r= -0.2).

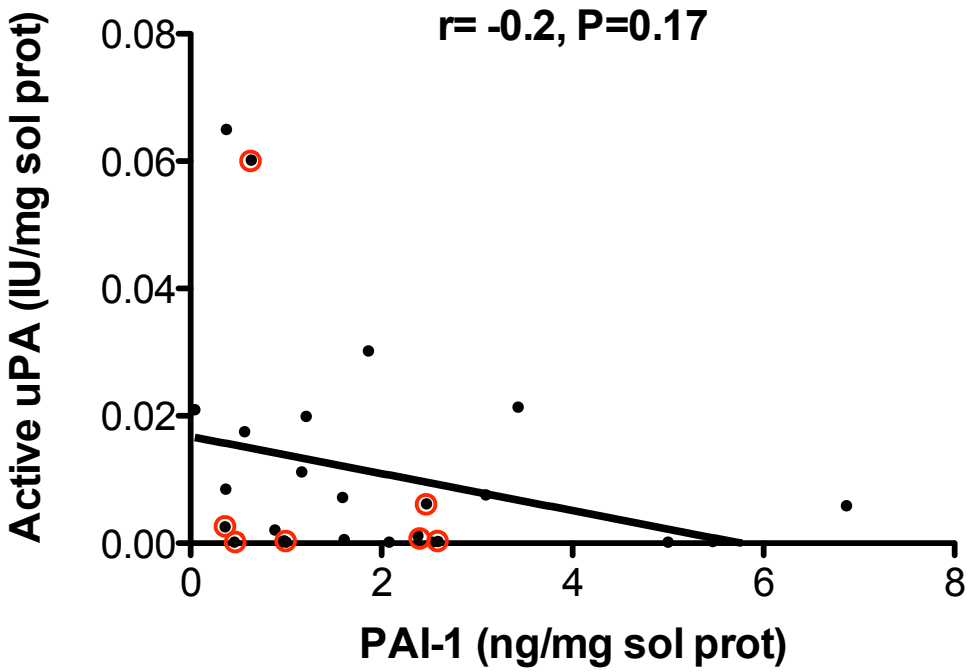


Figure 31: Correlation between active uPA and PAI-1 in tissue homogentaes from all venous ulcers. Points in red circles represent samples taken from healing venous ulcers

5.6 Discussion

It has been suggested that uPA in venous ulcers has a role in ECM degradation, either directly through activation of plasmin[188] or indirect by activating MMPs[80]. Our results show that there was no difference in the amount of total uPA protein and PAI-1 present in exudates obtained from healing and non-healing venous ulcers. Both uPA and PAI-1 were measurable in all tissue homogenates, with no statistical difference between healing and non-healing ulcers. This may suggest a generally increased fibrinolytic response within venous ulcers[112], but based on our results it is not possible to suggest a direct role for uPA or PAI-1 in ulcer healing.

A possible role of uPA may be the cleavage of suPAR between DI and DII-III. Cleavage of uPAR at the linker region between DI and DII-III can be induced by a variety of proteases in vitro[147], but so far only uPA can cleave this region in vivo[146]. Again as there was no difference in uPA between healing and non-healing ulcers it is not possible to speculate that this may be a role of uPA which may lead to healing of a venous leg ulcer.

PAI-1 is known to influence a variety of cellular functions[189] e.g. cell motility and angiogenesis that are important processes in wound healing[93, 96]. Studies using animal models have shown that PAI-1 is required during early wound healing especially during cell migration[190]. The finding of lower levels of PAI-1 in AWF compared with both healing and non-healing ulcer exudates is consistent with that of a previous study[81]. Angiogenesis is pivotal for normal wound healing[93] and in murine model PAI-1 has been shown to inhibit angiogenesis at higher doses, while promoting angiogenesis at a lower doses[191]. It is therefore possible to speculate that, PAI-1 has a pro-angiogenic role at lower concentrations found in acute wounds and an anti-angiogenic role at the higher concentrations of the chronic wound environment. This will however need further studies to confirm this hypothesis.

Our results comparing healing and non-healing venous ulcers have not shown a significant difference in the levels of PAI-1, in both tissue homogenates and the wound exudates. Based on our results we cannot draw firm conclusions on a role for PAI-1 in ulcer healing. Also we were unable to differentiate the active and inactive/latent forms of PAI-1 as the ELISA kit we used for determination of

PAI-1 measured both form and could not differentiate between the two forms of PAI-1.

The results of PAI-1 in tissue homogenates and wound exudates were contrasting as tissue homogenates from non-healers had higher levels of PAI-1 compared with tissue homogenates from healers whereas healing wound exudates had higher levels of PAI-1 compared with non-healing exudates. This may be because of the increased presence of vitronectin within the tissue matrix, to which PAI-1 can bind and stabalize [192].

No uPA activity was detected in any ulcer exudate or wound fluid analysed. Non-healer tissue homogenates had higer uPA activity compared to healers but this was not statistically significant. Active uPA has been found in the granulation tissue and ulcer edges of healing wounds[143, 177]. Active uPA may be affected by PAI-1 but we did not find any correlation between active uPA and PAI-1. This however was not an ideal assessment, as the method we used for detecting PAI-1 could not differentiate between active and latent form of PAI-I. Therefore further studies to analyze the role of active PAI-1 on uPA activity would clarify its role in venous ulcer healing.

Our aim was to assess if healing venous exudates and AWF showed similar levels of the plasminogen components. There was no similarity between healing ulcer exudates and AWF with respect to total uPA and PAI-1. Comparisons between AWF and chronic venous ulcers are also difficult to interpret because of the small number of AWF samples available, which makes a Type-II statistical error a distinct possibility.

Chapter 6: Cell migration in response to suPAR

6.1 Introduction

Wound healing is defined as complete re-epithelialisation of an epithelial defect. In order to achieve this keratinocytes at the ulcer edge must migrate towards the centre of the wound. The peptide 88-92 (SRSRY) present at the linker region between domains I and II of uPAR is highly chemotactic for the monocytes cell line, THP-1[154] and human embryonic kidney cells[170]

We therefore aimed to determine whether the SRSRY chemotactic fragment of suPARII-III would affect chemotaxis of adult human keratinocytes (HEKa).

Initially migration of THP-1 cells (immortal human monocytic cell line) was analysed in vitro using commercially prepared chemotactic peptide 88-92 to validate our experimental conditions. This was followed by analysis of migration of HEKa in response to chemotactic peptide, control (scrambled) peptide and the wound fluids in which levels of suPAR fragments have already been measured.

6.2 Methods

Initial experiments were carried out using THP-1 cells to validate the chemotactic efficacy of the SRSRY peptide in our hands. This was followed by analysis of the effect of the peptide and wound fluids on migration of HEKa.

All procedures were carried out under sterile conditions and materials obtained from Invitrogen unless otherwise indicated. Primary adult Human Epidermal Keratinocytes (HEKa; C-005-5C) were used for all experiments involving keratinocytes. Coating matrix containing type I collagen (R-011-K) was used to coat the flasks/wells 30mins prior to cell culture. EpiLife Medium (M-EPI-500-CA) with EDGS (EpiLife defined growth supplement; S-012-5) was used as cell culture supplement media. THP-1 cells (88081201) were obtained from HPA culture collections and cultured in Roswell Park Memorial Institue medium (RPMI; R7509; Sigma) containing 10% heat inactivated foetal calf serum (FCS; F7524, Sigma) and L Glutamine (25030; Gibco).

Cell viability was measured using trypan blue (T8154; Sigma). Cell suspensions were diluted in equal amounts with trypan blue (1:1) and non-stained (viable) cells were counted using a haemocytometer.

6.2.1 Cell culture for HEKa

All steps of cell culturing were performed in the cell culture fume hood except centrifugation and cell counting using haemocytometer.

1. Growth supplement (5ml) was added to 500ml of EpiLife medium, pre-warmed in a water bath at 37°C.
2. The medium was sterilized by passing through Naglene filter (0.45μm pore size; Thermo Scientific) after which 5ml of an antibiotic mixture (15640-055) consisting of penicillin (5mg/ml), streptomycin (5mg/ml) and neomycin (10mg/ml) was added to it.

   75cm^2 flask - 15ml of supplement

   25cm^2 flask - 5ml of supplement

   24-well plate - 0.35ml of supplement

3. A 75cm^2 flask was coated by adding 50μl coating matrix (R-011-K), diluted in 5ml dilution media from the coating matrix kit. The flask was rocked to ensure uniform distribution. Flasks were capped and incubated for 30mins at room temperature. Excess coating matrix was removed and the flasks used immediately or stored at 2 to 8 °C for up to 6wks.

4. The vial of keratinocytes was removed from the freezer and thawed in a 37°C water bath and viability assessed by taking a 20μl aliquot of the cell suspension and adding 20μl of trypan blue. The dye cell suspension was mixed and a small aliquot placed on Neubauer haemoctytometer (Hawksley). The number of cells containing trypan blue were enumerated using an inverted microscope (Olympus).

5. The contents of the vial were diluted in supplemented medium to obtain 2.5×10^4 viable cells per ml added to $75cm^2/25cm^2$ culture flasks. The flasks were then swirled to achieve even distribution of the medium and the cells incubated at 37°C with 5% CO2/95%.

6. Approximately half of the medium in the flask was removed and replenished after 48hrs. Subsequently, similar media changes were carried out every 24hrs.

6.2.2 Dissociation of HEKa cells from the flask for subculturing and use

1. Culture media was removed from the flasks and placed in a sterile tube to be used later for neutralisation of the trypsin EDTA solution used to disassociate cells from the flask.
2. 1 ml of trypsin/EDTA (0.025% trypsin, 0.01% EDTA in PBS) was added to 25cm flask (2-3ml for 75 cm flask). Flasks were rocked gently so that the whole surface was covered followed by aspiration of all trypsin/EDTA after 1min (60 seconds) and incubation at 37°C with 5% CO2 for a further 5mins.
3. The sides of the flask were tapped gently to dislodge cells, 3ml of culture media (9ml for 75cm) was added to neutralise the trypsin/EDTA and the cell supension transferred to a 50ml falcon tube.
4. The flask was washed with a further 3ml of culture media (9ml for 75cm), which was then decanted into the falcon tube containing the cells.

5. The cell suspension was centrifuged at 180g for 7mins and the supernatant was carefully removed without disturbing the cell pellet at base.
6. The cell pellet was resuspended in 3ml of culture media and counted using the haemocytometer as described above. Cells were either sub-cultured or used in experiments.

6.2.3 Cell culture and dissociation of THP-1 cells

All steps of cell culturing were performed in the cell culture fume hood except centrifugation and cell counting using haemocytometer.

1. Cell culture media was prepared by adding 50ml of 10% heat inactivated FBS and 5ml of 2mM L-Glutamine to 445ml of RPMI media that was pre-warmed in a water bath at 37°C.
2. The media was sterilized by passing through Naglene filter (0.45μm pore size; Thermo Scientific). The appropriate volume of freshly prepared media is added to culture flasks.

   75cm^2 flask - 25ml of media

   175cm^2 flask - 50ml of media

3. The vial of THP-1 cells was removed from the freezer and thawed in a 37°C water bath. Cell viability was assessed by taking 20μl aliquot of the cell suspension and adding 20μl of trypan blue. The dye cell suspension was mixed and a small aliquot placed on Neubauer haemocytometer

(Hawksley). The number of cells containing trypan blue (dead cells) was enumerated using an inverted microscope (Olympus).

5. The contents of the vial were diluted by addition of the prepared culture media. Cells were then put into the culture flasks at concentration of 3×10^5 viable cells/ml. The culture flasks were then placed in the incubator at 37°C with 5% CO2
6. Cells were allowed to grow in the culture media till the concentration reached 1×10^6 cells.
7. For subculturing the cell culture media containing the cells was placed in a 50ml falcon tube. This was centriguged at 100g for 5 mins. After spinning the supernatant fluid is carefully aspirated without disturbing the cell pellet. Cellular pellet was then resuspended in 1ml of media and counted as before using a haemocytometer.
8. This was followed by a further subculture or cells were frozen for storage. Cellular concentration for storage was 6×10^6 THP-1 cells/ml.

6.2.4 Chemotaxis analysis using the Boyden Chamber

A modified Boyden chamber, with an 8μm pore membrane separating the two chambers (BD 351164), was used for analysis of chemotaxis. Cells from a culture flask were spun (at 180g for 7mins for HEKa & at 100g for 5mins) resulting in formation of a cell pellet. This was resuspended in 1ml of culture media. Calcein AM (Invitrogen, C1430) at 10μM concentration was added to 1ml of this culture media containing the required cells. After a 3minute interval, 5ml of serum free media was added to wash away excess calcein. This suspension

was then centrifuged again at 180g for 7minutes to reform the cell pellet. Required number of cells were taken and pipetted in the top wells of 96-well modified Boyden chamber fluoroblok plates (~20,000 cell/well for HEKa and ~50,000 cells/well for THP-1).

During the course of the experiments optimal coating with ECM proteins (collagen, fibronectin and vitronectin) was also determined. Chemoattractants and serum free media (EpiLife) as negative control was placed in the bottom wells. Each sample was measured in triplicates. Fluorescence was measured in the bottom chambers with the excitation and emission filters set at 485nm and 535nm respectively on a Victor fluorometer (Perkin Elmer). Each well was read for 0.5seconds. For keratinocyte migration reading were taken at 40min intervals going up to 4hrs. For THP-1 cells readings were taken at 20min intervals going up to 2hrs. The effect of treatment on cell chemotaxis was taken as the ratio of fluorescence intensity in the treatment well to that of the negative control. All analysis were performed using two way ANNOVA with Bonferroni post-test using PRISM Ver5.

(i) SRSRY peptide as a chemotactic agent: Commercially prepared five amino acid chemotactic peptide sequence, similar to that found within suPARII-III serine-argenine-serine-arginine-tyrosine (SRSRY) was obtained from BaChem, UK. In addition a scrambled amino acid sequence with same five amino acids but different sequence was obtained from BaChem, UK and used as a control peptide. Chemotactic potential of HEKa and THP-1 cells was assessed by placing the cells in the upper

chambers and the chemotactic or control peptide in the lower chamber of boyden chamber. All experiments were performed in triplicate

(ii) Effects of coating with different ECM proteins -. The membrane containing top well of the Boyden chamber was coated with one of the ECM proteins, collagen typeI (Sigma C8919), fibronectin (PHE0023) or vitronectin (PHE0011). Desired ECM protein (collagen, fibronectin or vitronectin) was diluted to 10μg/ml in sterile PBS, which was then placed in the bottom wells (200μl/well). Top wells were then inserted into the bottom wells and incubated at room temperature for 2-3hrs. This was followed by a wash with PBS after which the top wells were used for the desired experiments. All experiments were performed in triplicates

(iii) Dilution effect of wound fluids - A pooled exudate was created from both healing and non-healing ulcer exudates. HEKa migration in response to various dilutions (2.5%, 5%, 10%, 15% and 20%) of pooled wound exudates that were placed in the lower chambers was observed. Wound fluid was diluted in SFM. All experiments were performed in triplicates

(iv) Healing and non-healing exudates – Migration of HEKa was observed in response to exudates obtained from healing (n=4) and non-healing (n=9) ulcers. As determined by the dilutional experiments all wound exudates (healer and non-healer) were at 5% dilution. All experiments were performed in triplicates

(v) Depletion of sUPAR - Depletion of suPAR was carried out on a pool of healing and non-healing exudates by affinity column chromatography as described in chapter3, section3.2.3. Migration of HEKa in response to depleted healing and non-healing ulcer exudates was assessed. All experiments were performed in triplicates

6.2.5 Scratch assay

Scratch assays were also used to assess keratinocyte migration. HEKa cells were incubated at 8000-cells/well in a 24-well plate coated with typeI collagen (Sigma C8919). Once the HEKa cells reached >80% confluence scratches were made in the cultured cells, in the centre of each well, using a sterile 200μl pipette tip, after which the media containing detached cells was aspirated and replaced with serum free media (SFM) with chemotactic peptide, SFM with scrambled peptide or SFM with varying concentrations of wound fluids from patients who had healing or non-healing venous ulcers, added. Plates were incubated at 37°C, 5% CO2/95% air humidified cell culture incubator and micrographs of each scratch taken at 0, 12 and 24hrs. All experiments were carried out in quadruplicate.

(i) Proliferation or migration

Assays were repeated in the presence of mitomycin C, which is a potent inhibitor of cell proliferation in order to determine whether any scratch coverage was the result of cell proliferation or cell migration. Mitomycin C (Sigma, M4287) at concentration of 4μl/ml was added to cell cultures. Experiments with and without Mitomycin C were carried out on 4 non-healing

and 4 healing ucler exudates. All experiments were carried out in quadruplicates.

(ii) sUPAR depletion

In order to determine whether suPAR has a role in in-vitro wound healing, scratch assays were repeated after depleting suPAR from the ulcer exudates by affinity column chromatography (as described in chapter3 section3.2.3). Experiments were performed with three healing and three non-healing ulcer exudates. One half of each ulcer exudate was depleted of suPAR and experiments were carried out with depleted and non-depleted ulcer exudates. All experiments were carried out in quadruplicates.

All statistical analysis were performed using two way ANOVA and Bonferroni post-test with PRISM ver5

6.3 Boyden chamber results

6.3.1 Optimization of experimental conditions

Initial migration studies were performed to validate experimental conditions. Commercial chemotactic peptide (SRSRY) induced a significantly higher migration in THP-1 cells at concentratin of 1picomole (1pM) and 1femtomole (1fM) compared to similar concentrations of control (scrambled) peptide after 60 mins ($p<0.0001$, Fig 32). There was no difference in the migration of THP-1 cells towards 1pM and 1fM of cheomactic peptide (Table 25)

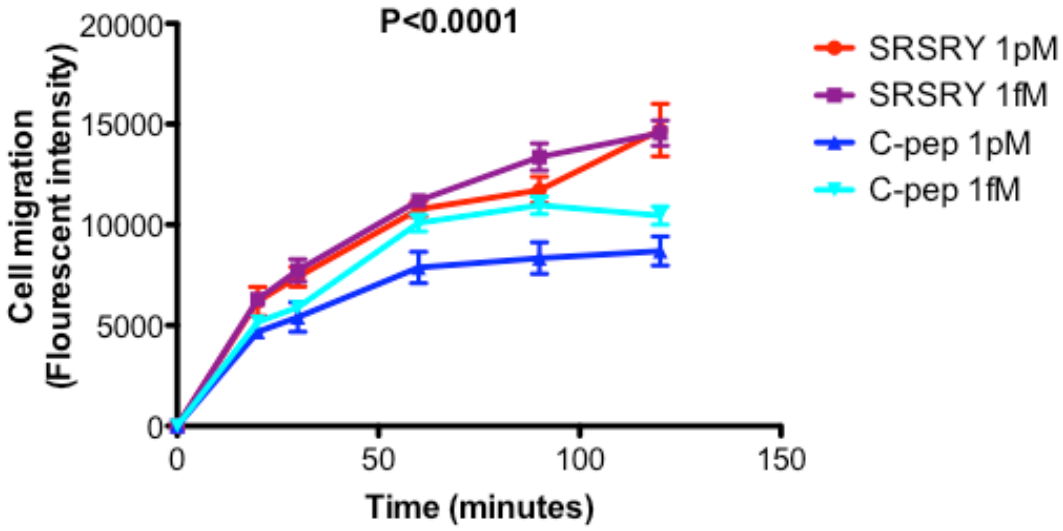


Fig 32: Migration of THP-1 towrds chemotactic (SRSRY) or control/scrambled peptide (C-pep). SRSRY induced a significantly higher migration of THP-1 cells compared to control. Statistical analysis using two-way ANNOVA. Experiments performed in triplicate. (see Appendix for mean and SEM valuses at each time point)

Table 25: Bonferroni post-test comparing THP-1 migratory response (difference in relative fluorescence intensity) to chemotactic peptide (SRSRY) and control peptide (C-pep). NS=not significant

Time (mins)	SRSRY- 1pM vs SRSRY- 1fM (P value)	SRSRY-1pM vs C-pep-1pM (P value)	SRSRY-1pM vs C-pep-1fM (P value)	SRSRY-1fM vs C-pep – 1pM (P value)	SRSRY-1fM vs C-pep – 1fM (P value)
0	NS	NS	NS	NS	NS
20	(NS)	(NS)	(NS	NS	NS
30	(NS)	(NS)	(NS)	(<0.05)	(NS)
60	(NS)	(<0.01)	(NS)	(<0.001)	(NS)
90	(NS)	(<0.001)	(NS)	(<0.001)	(<0.05)
120	(NS)	(<0.001)	(P<0.001)	(P=0.001)	(<0.001)

6.5.1 Migartion of HEKa towards chemotactic peptide

We then aimed to see if HEKa cells migrated towards the chemotactic peptide. We also analysed the optimal concentration of chemotactic peptide required for inducing HEKa migration. Results from these studies showed that chemotactic peptide at concentration of 1 picomole (pM) induced a better migrational response when compared with other concentrations of chemotactic peptide (Fig 33, P=<0.0001). Also 1pM concentration showed better differentiation between chemotactic peptide and control peptide (Table 26). These results proved that HEKa migration can be induced with the chemotactic peptide sequence (SRSRY) that is present on suPARII-III fragment

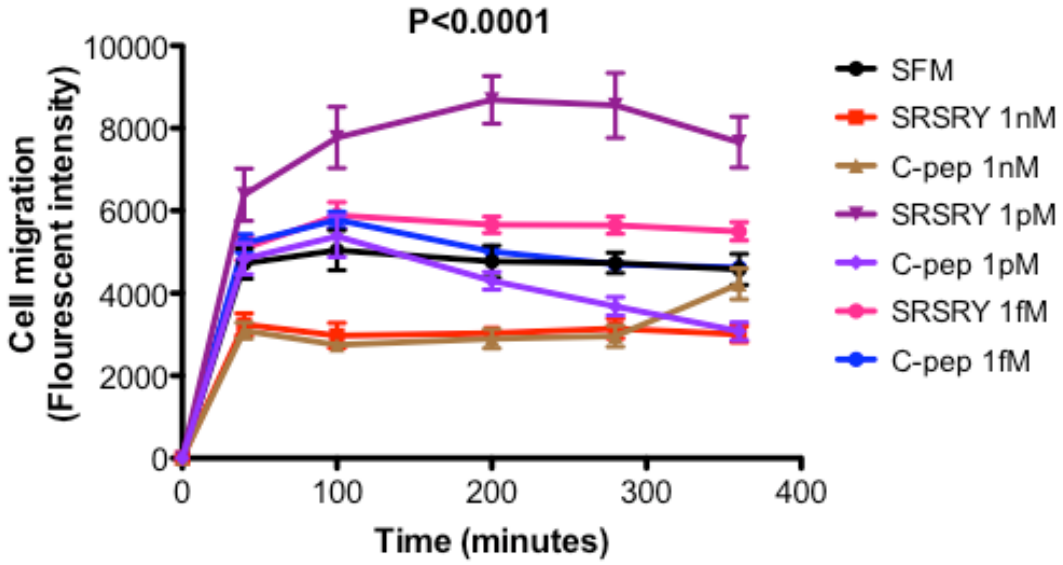


Fig 33: Migration of HEKa towards chemotactic (SRSRY) or control/scrambled peptide (C-pep). Chemotactic peptide (SRSRY) induced a significantly higher migration of HEKa at concentration of 1picomole compared to control peptide. SRSRY-pep= Chemotactic peptide. C-pep= Control peptide. Statistical test using two-way ANNOVA. Experiments performed in triplicate. (See Appendix for mean values and SEM at each time point)

Table 26: Bonferroni post-test comparing migratory response of HEKa (difference in relative fluorescence intensity) in response to various concentrations of chemotactic peptide (SRSRY), SFM (serum free media) and control peptide (C-pep). NS=not significant.

Time (mins)	SRSRY-1pM vs SFM (P value)	SRSRY-1pM vs SRSRY-1nM (P value)	SRSRY-1pM vs SRSRY-1fM (P value)	SRSRY- 1pM vs C-pep 1fM (P value)	SRSRY-1pM vs C-pep 1pM (P value)
0	NS	NS	NS	NS	NS
40	<0.001	<0.001	<0.05	NS	<0.01
100	<0.001	<0.001	<0.001	<0.001	<0.001
200	<0.001	<0.001	<0.001	<0.001	<0.001
280	<0.001	<0.001	<0.001	<0.001	<0.001
360	<0.001	<0.001	<0.001	<0.001	<0.001

6.5.2 Migration of HEKa in the presence of ECM proteins

We also optimize HEKa migration in the presence of various ECM proteins that can be found in the environment of a healing wound. Migration of HEKa in the presence of collagen (CN), fibronectin (FN) and vitronectin (VN) was analysed. At 1pM concentration HEKa cells showed significantly higher migration towards chemotactic peptide compared to the scrambled peptide (Fig 33). There was however no significant difference between uncoated, CN coated, FN coated and VN coated wells at 1pM concentration except at 160mins, which returned to a non-significant level at 200mins (Table 27)

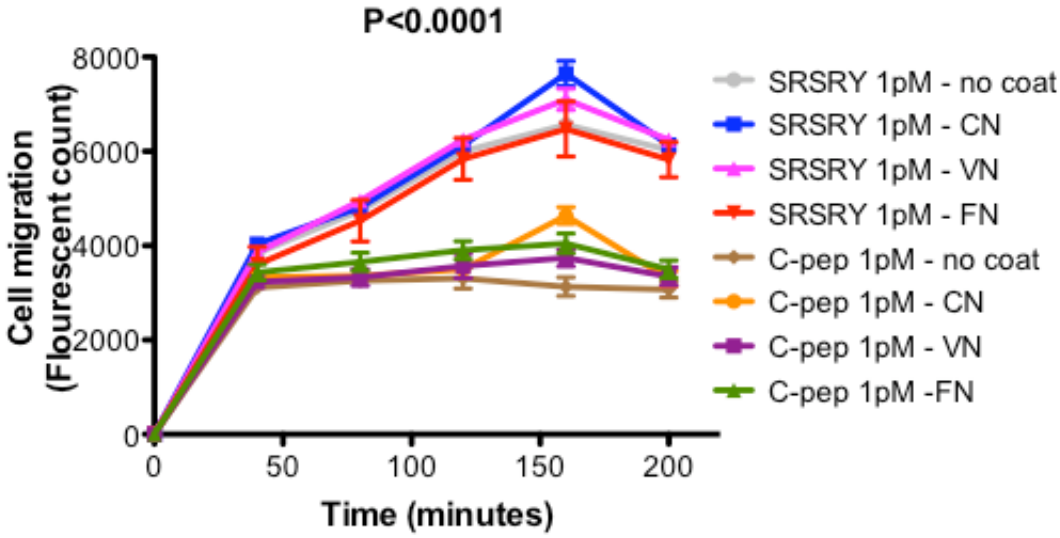


Fig 34: HEKa migration towards chemotactic (SRSRY) or control peptide (C-pep) in the presence of various ECM proteins. HEKa migration was higher at a concentration of 1picomole and in presence of collagen (CN) compared to the presence of no coating, vitronectin (VN) and fibronectin (FN). Statistical anlaysis using two way ANNOVA. Experiments performed in triplicate. (See Appendix for mean and SEM values at each time point)

Table 27: Bonferroni post-test comparing migratory response of HEKa cells towards the chemotactic peptide in presence of various ECM proteins (difference in relative fluorescence intensity). NS=not significant. CN=collagen. VN=vitronectin. FN=fibronectin

Time (mins)	SRSRY-no coat vs SRSRY-CN (P value)	SRSRY-no coat vs SRSRY-VN (P value)	SRSRY-no coat vs SRSRY-FN (P value)	SRSRY-CN vs SRSRY-VN (P value)	SRSRY-CN vs SRSRY-FN (P value)	SRSRY-VN vs SRSRY-FN (P value)
0	NS	NS	NS	NS	NS	NS
40	NS	NS	NS	NS	NS	NS
80	NS	NS	NS	NS	NS	NS
120	NS	NS	NS	NS	NS	NS
160	<0.001	<0.05	NS	NS	<0.001	<0.05
200	NS	NS	NS	NS	NS	NS

6.5.3 Optimal wound fluid dilution required for HEKa migration

Optimal concentration of wound fluids required to induce HEKa migration was also analysed. Wound fluid dilution of 2.5%, 5% and 10% showed a better migration compared to dilutions at 15% and 20% (Fig 35, Table 28b). There was no statistically significant difference between 2.5%, 5% and 10% wound fluid dilution (Bonferroni post test $P<0.05$, Table 28a). Based on this result a dilution of 5% was used for all boyden chamber migration assays involving wound fluids

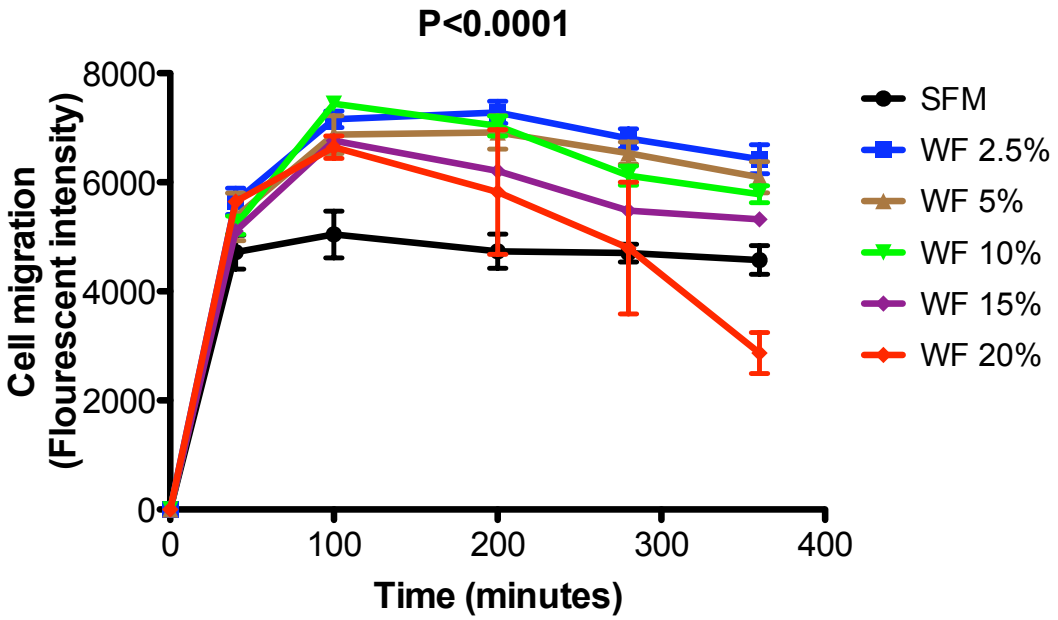


Fig 35: Migration of HEKa towards various dilutions of wound fluids. Various dilutions of wound fluids had a statistically significant effect in the overall migrational variation. WF= wound fluids. Statistical analsis by two way ANNOVA. Experiments performed in triplicates. (See Appendix for mean and SEM values at various time points)

Table 28a: Bonferroni post-test comparing migratory response of HEKa towards various dilutions of wound fluids (difference in relative fluorescence intensity). NS= not significant. WF= wound fluid.

Time (mins)	WF-2.5% vs WF-5% (P value)	WF-2.5% vs WF-10% (P value)	WF-2.5% vs WF-15% (P value)	WF-2.5% vs WF-20% (P value)	WF-5% vs WF-10% (P value)
0	NS	NS	NS	NS	NS
40	NS	NS	NS	NS	NS
100	NS	NS	NS	NS	NS
200	NS	NS	NS	<0.05	NS
280	NS	NS	<0.05	<0.001	NS
360	NS	NS	NS	<0.001	NS

Table 28b: Bonferroni post-test comparing HEKa migratory response towards various dilutions of wound fluids (difference in relative fluorescence intensity). NS= not significant. WF= wound fluid

Time (mins)	WF-5% vs WF-15% (P value)	WF-5% vs WF-20% (P value)	WF-10% vs WF-15% (P value)	WF-10% vs WF-20% (P value)	WF-15% vs WF-20% (P value)
0	NS	NS	NS	NS	NS
40	NS	NS	NS	NS	NS
100	NS	NS	NS	NS	NS
200	NS	NS	NS	NS	NS
280	NS	<0.01	NS	<0.05	NS
360	NS	<0.001	NS	<0.001	<0.001

6.5.4 Migration of HEKa towards healing and non-healing wound exudates

HEKa migration was compared between healing and non-healing wound exudates. Both healing and non-healing wound fluids caused a variable migratory effect on HEKa cells. There was no differential migration of HEKa towards healing or non-healing exudates. Some of the non-healing exudates even resulted in higher HEKa migration compared to healing exudates (Fig 36). In order to see a differential effect of healing or non-healing wound exudates upon HEKa migration the mean of healing and non-healing exudate results were plotted, which again did not show a significant difference in HEKa migration between healing and non-healing exudates (Fig 37).

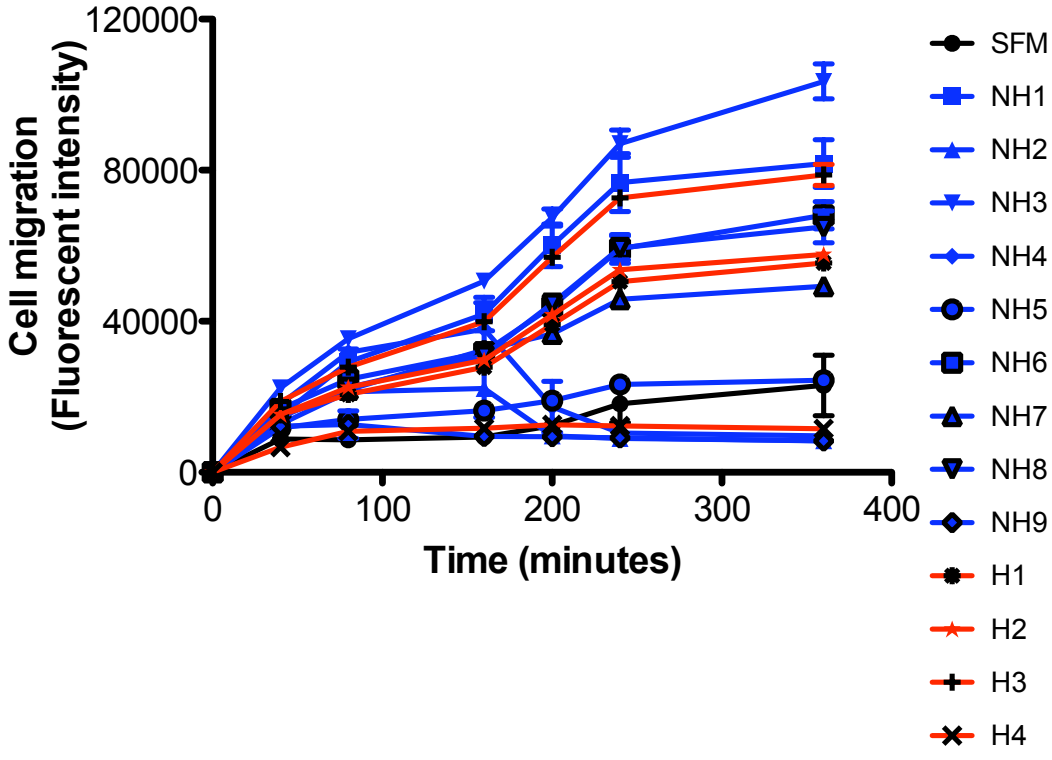


Fig 36: Migration of HEKa towards healing (n=4) and non-healing (n=9) wound exudates. Statistaical analysis by two way ANNOVA. Experiments performed in triplicates

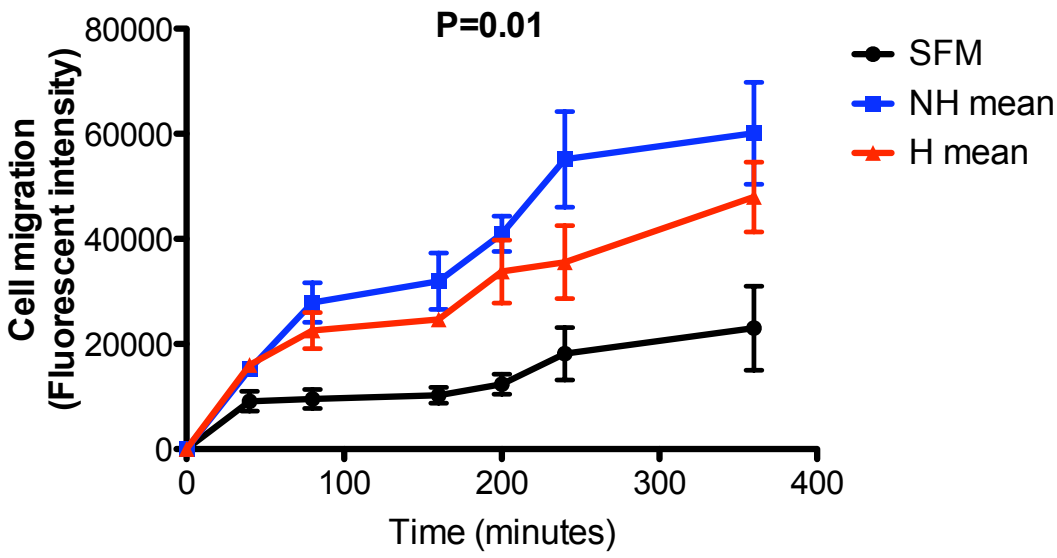


Fig 37: Migration of HEKa towards healing (mean) and non-healing (mean) exudates. NH= non-healing ulcer exudate. H= healing ulcer exudate. Statistical analysis by two way ANNOVA

6.5.5 Migration of HEKa towards depleted wound exudates

To analyze the role of suPAR in HEKa migration boyden chamber assays were repeated using depleted healing and non-healing wound exudates. Pools of healing and non-healing wound fluids were depleted off suPAR as previously described in chapter 3, using affinity column chromatography. These results showed a higher migration of HEKa towards the depleted non-healing wound exudates when compared to the depleted healing exudates but the results were not statistically significant using Bonferroni post test (P>0.05, Fig 38)

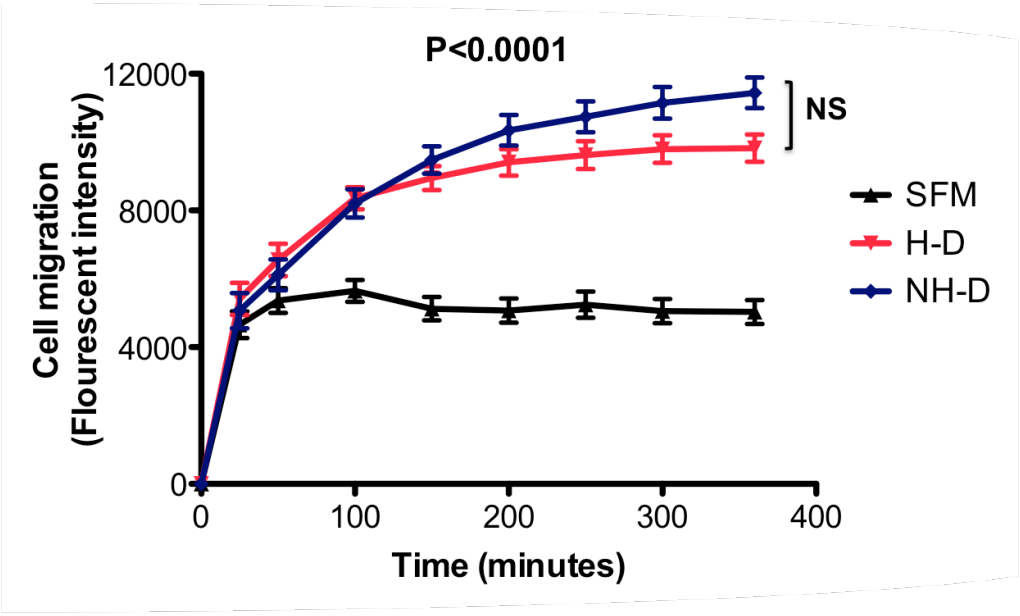


Fig 38: Migration of HEKa towards depleted wound exudates. H-D=depleted healing exudate, NH-D=non-depleted healing exudate. Statistical analysis by two way ANNOVA. Experiments peromed in triplicates. There was overall significant difference using two way ANNOVA, however there was no statistically significant difference between the depleted healers and depleted non-healers using bonferroni post test.

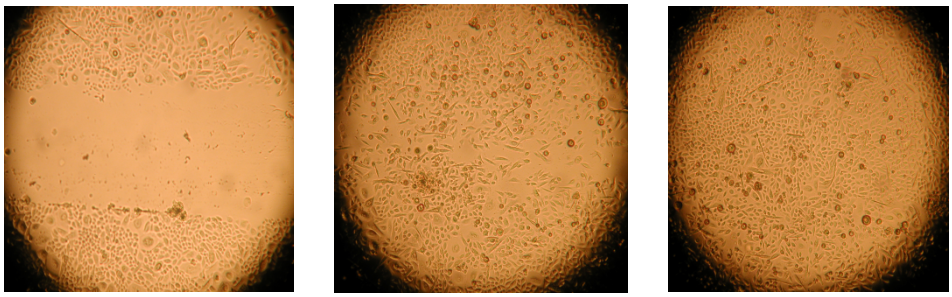
6.4 Scratch assay results

6.5.6 Optimization of wound exudate concentrations

Initial experiment using 2% wound exudates resulted in cell death in all cells treated with both healing and non-healing wound exudates. Scratch assays were then repeated using 0.5% of both healing and non-healing wound exudates. This resulted in complete healing of scratch in the healers whereas the non-healers resulted in cell death at end of 24 hrs (Fig 39,40). Quantitative analysis of scratch coverage between the two groups showed

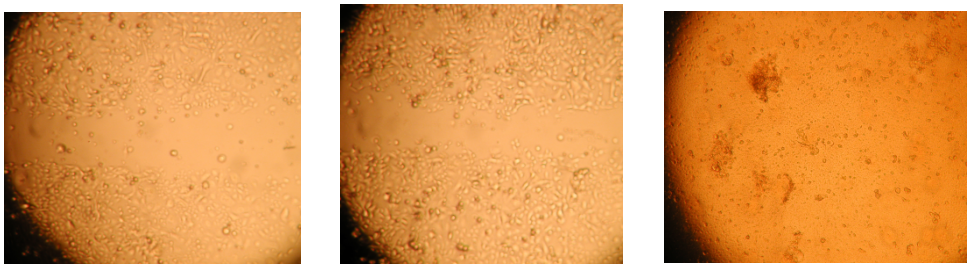
significantly increased coverage of the scratch in the presence of healing exudates (P value 0.006, Fig 41)

Fig 39: Coverage of scratch after treatment with 0.5% healing exudate. Complete healing of scratch by 24 hours



0 hour 12 hrs 24 hrs

Fig 40: Coverage of scratch after treatment with 0.5% non-healing exudate. Cell death at 24 hours



0 hour 12 hrs 24 hrs

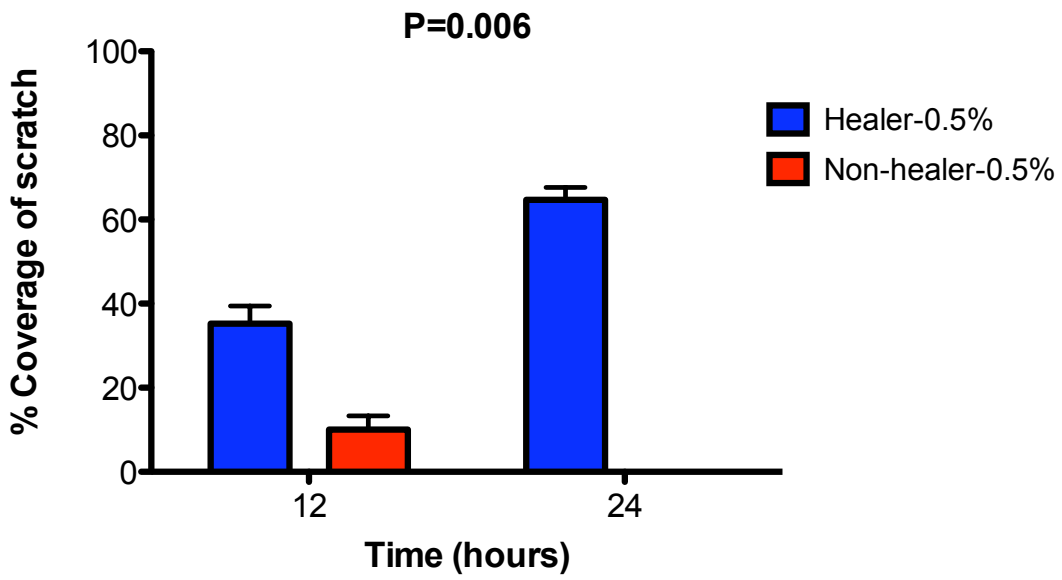
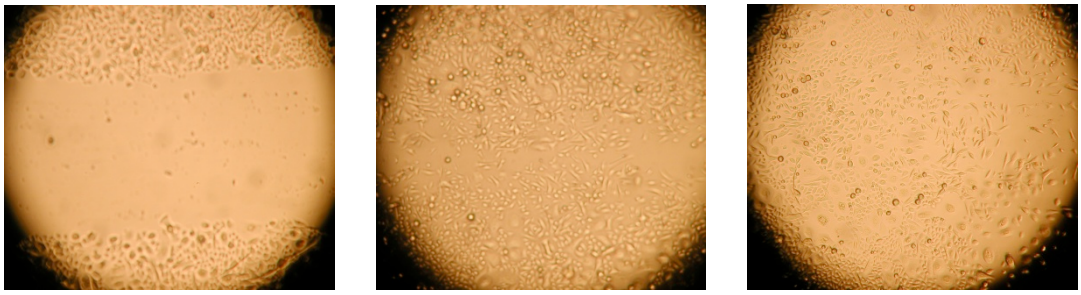


Fig 41: Comparison of scratch coverage after treatment with 0.5% healing and non-healing wound exudates. Statistical analysis using two way ANNOVA. Experiments performed in quadruplicates.

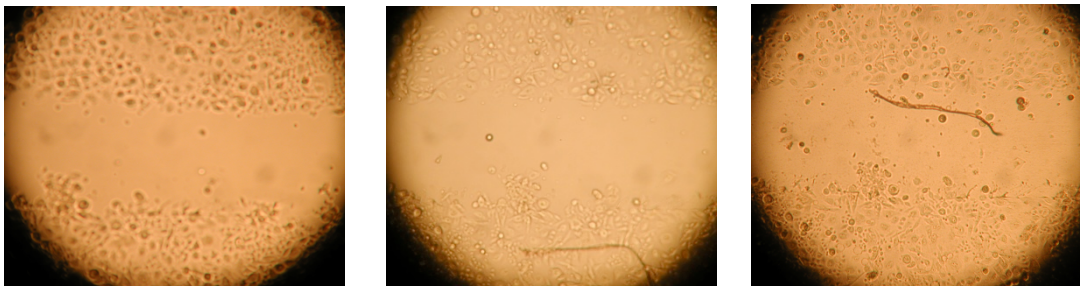
Repeating the experiments with 0.1% non-healing wound exudates did not result in cell death (Fig 42,43). Cells treated with healing exudates still resulted in a significantly higher coverage of scratch as compared to cell treated with non-healing wound exudates (P=0.008, Fig 44)

Fig 42: Scratch coverage after treatment with 0.1% healing exudate. Complete healing at 24hours.



0 hour 12 hrs 24 hrs

Fig 43: Coverage of scratch after treatment with 0.1% non-healing wound exudate. Incomplete healing of scratch at 24hours.



0 hour 12 hrs 24 hrs

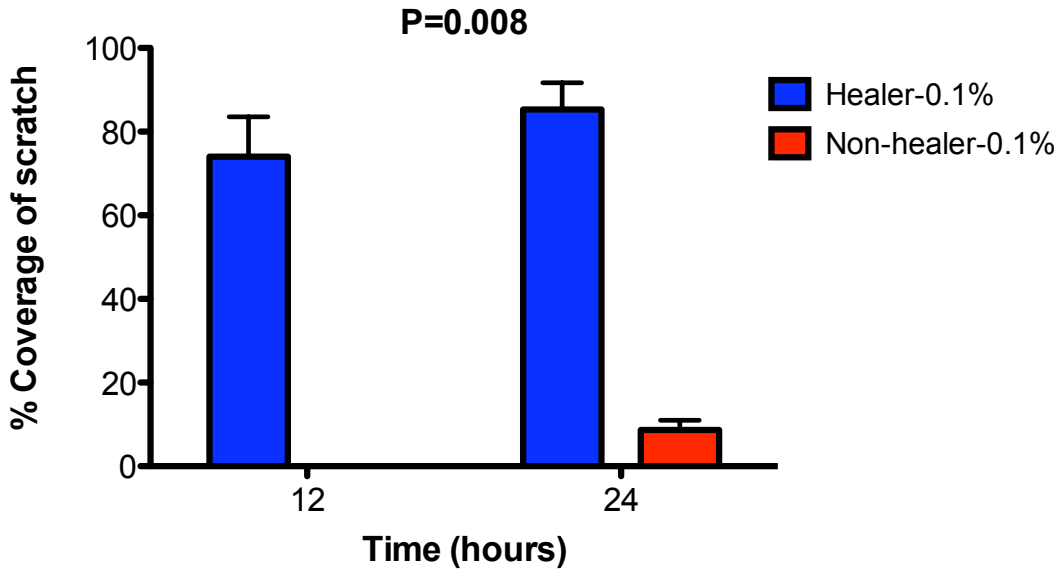
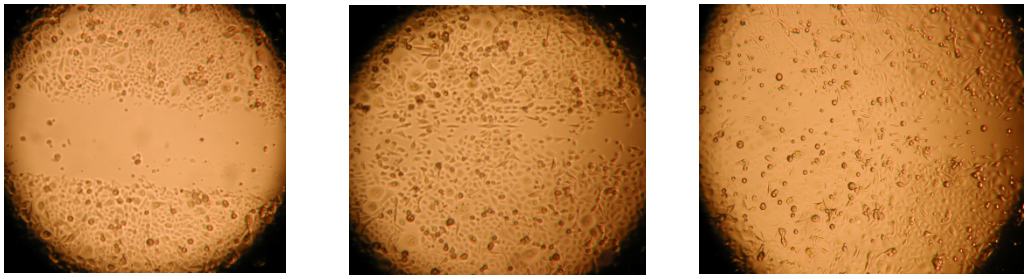


Fig 44: Comparison of scratch coverage after treatment with 0.1% healing and non-healing wound exudates. Statistical analysis using two way ANNOVA. Experiments performed in quadruplicates.

6.5.7 Scratch coverage in presence of Miotmycin C

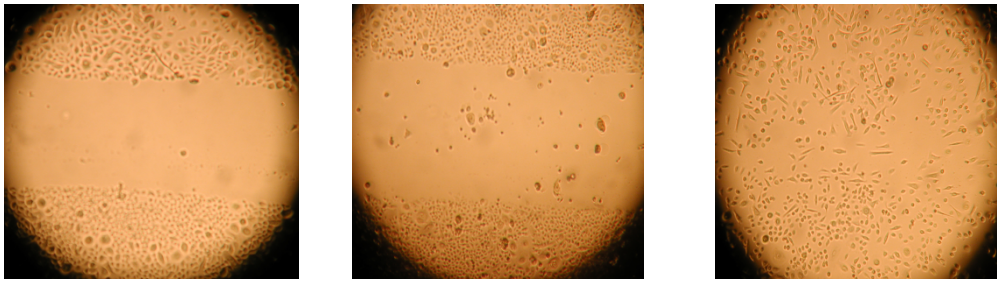
Scratch coverage could have been due to both cell proliferation and cell migration. In order to clarify the role of cell migration in coverage of the scratch assays were repeated in presence of Mitomycin C, which is a potent inhibitor of cell proliferation. There was no statistically significant difference in scratch coverage after treatment with or without Mitomycin C (Fig 45-47, Table 24, $P<0.05$)

Fig 45: Scratch coverage after treatment with 0.1% healing exudate in presence of Mitomycin C



0 hour 12 hrs 24 hrs

Fig 46: Scratch coverage after treatment with 1% non-healing exudate in presence of Mitomycin C



0 hour 12 hrs 24 hrs

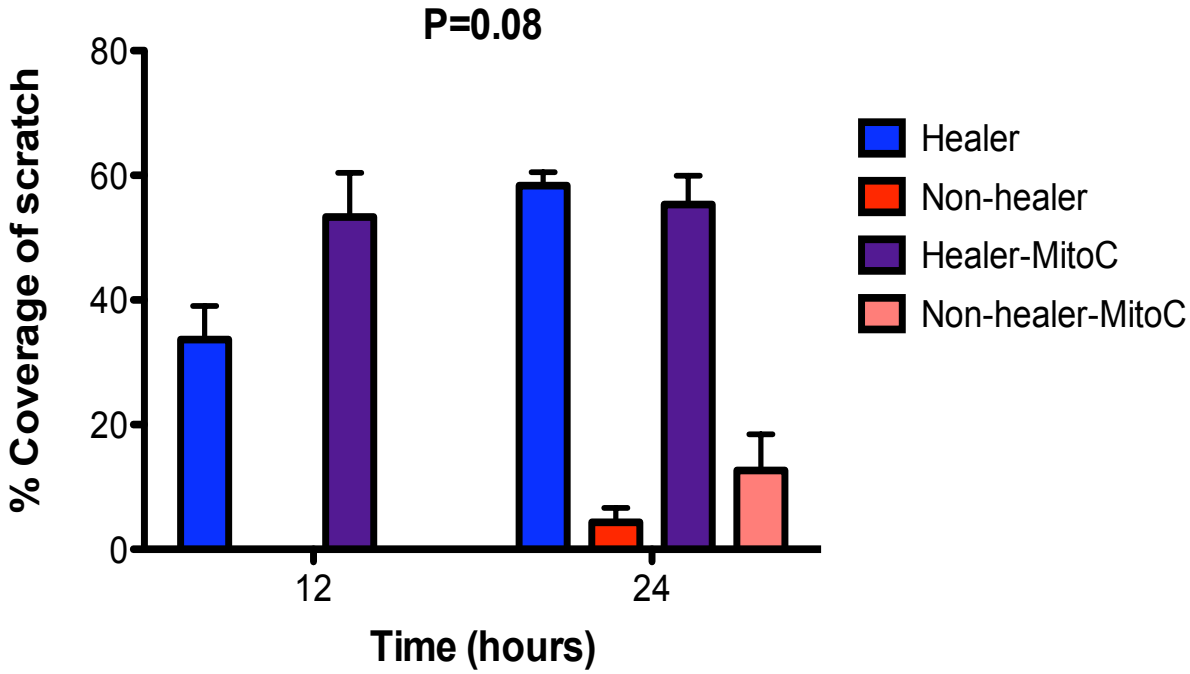


Fig 47: Comparison of scratch coverage with 0.1% healing (n=4) and 1% non-healing (n=4) ulcer exudates in presence of Mytomycin C. There was no statistically significant difference in the presenc or absence of Mitomycin C. Statistical analysis using two way ANNOVA. Experiments performend in quadruplicate

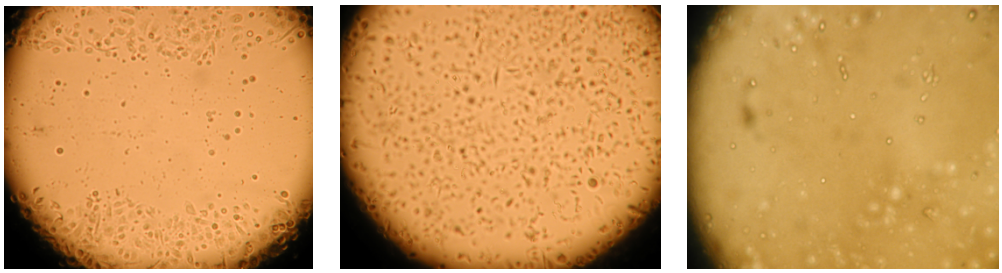
Table 29: Bonferroni post-test comparing scratch coverage with 0.1% ulcer exudates in presence and absence of Mitomycin C. No difference in scratch healing in presence or absence of Mitomycin C. NS=not significant

Time (hrs)	Healer vs Non-healer (P value)	Healer vs Healer-MitoC (P value)	Non-healer vs Non-healer-MitoC (P value)
0	NS	NS	NS
12	<0.001	<0.01	NS
24	<0.001	NS	NS

6.5.8 Scratch coverage after depletion of suPAR

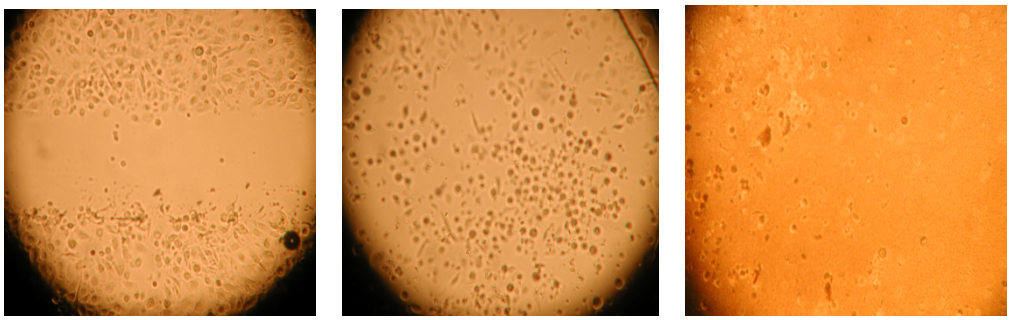
In an attempt to clarify the role of suPAR in in-vitro wound healing, scratch assays were repeated after depleting the wound exudates off suPAR as previously done during validation using affinity column chromatography. Treatment of scratch with suPAR-depleted wound exudates from both healing and non-healing ulcers resulted in cell death (Fig 48,49). Images at 12 hour showed cells that were floating after being lifted off the basement. At 24 hrs cell death was obvious as there was complete loss of cellular morphology. Expression of Annexin-V, a marker of apoptosis was expressed highly in the keratinocytes treated with suPAR-depleted exudates compared with non-depleted exudates (Fig 50).

Fig 48: Coverage of scratch after treatment with suPAR depleted healing exudate



0 hour 12 hrs 24 hrs

Fig 49: Scratch coverage after treatment with suPAR depleted non-healing exudate



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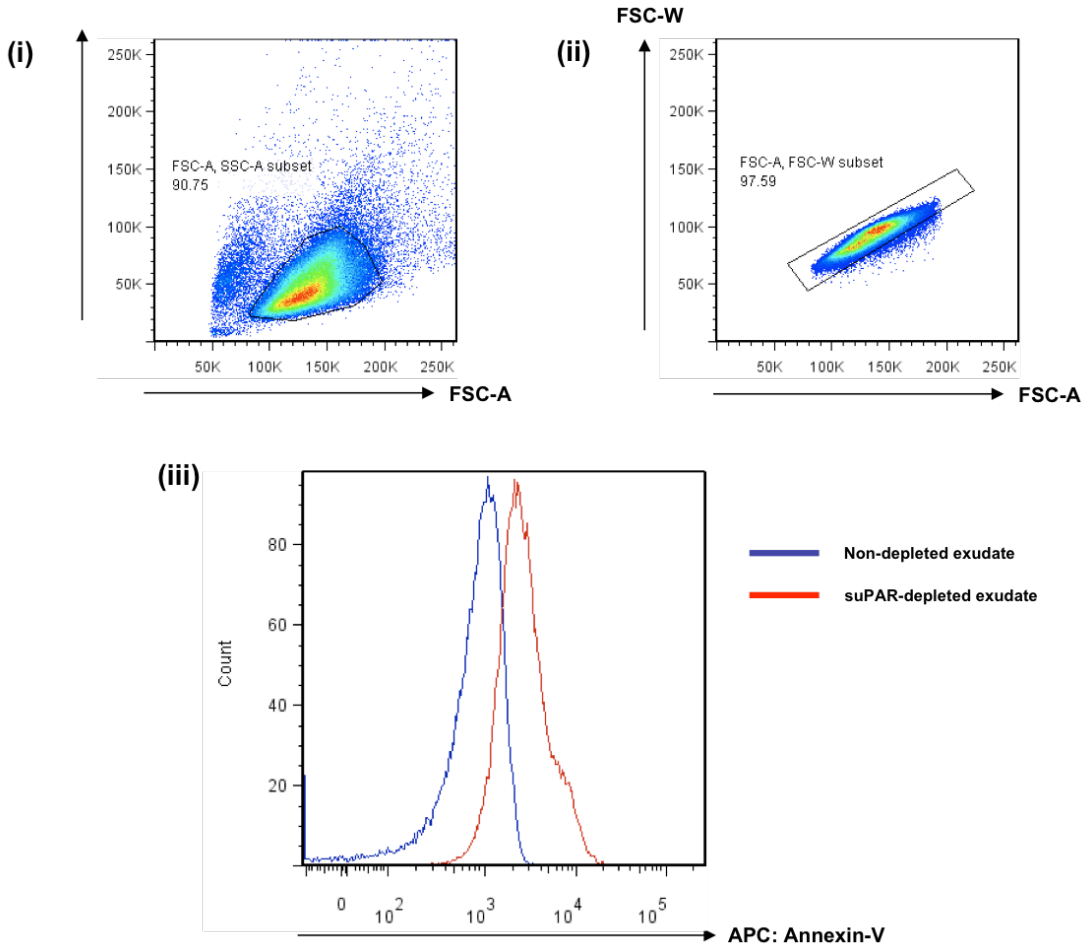

Fig 50: Effect of suPAR depletion on HEKa cell viability. (i) Keratinocytes gated according to size and scatter properties with (ii) doublets excluded. (iii) Typical histograms showing higher expression of Annexin-V in keratinocytes cultured with suPAR-depleted exudate (red) compared with non-depleted exudate (blue)

6.6 Discussion

The commercial SRSRY peptide that is found on suPARII-III was chemotactic for human adult keratinocytes compared with a scrambled sequence of this peptide. The migration of HEKa towards all exudates was higher than control (SFM). Comparison between healers and non-healers revealed no significant difference in HEKa chemotaxis. Variability was high, with a substantially greater HEKa migratory response towards some non-healing ulcer exudates that had lower levels of suPARII-III. This suggests that suPARII-III may not be a primary chemotactic stimulant to in-vitro HEKa migration.

This possibility was further proven when suPAR was depletd from the ulcer exudates. The difference between depleted non-healing ulcer exudates and depleted healing ucler exudates did not increase and remained similar to non-depleted levels. Possible reasons for the poor chemotactic effect of the ulcer exudates could include the lack of other facilitative cell surface proteins such as integrins and GCRP that are required by suPAR to influence intracellular signaling and migration[152].

The lack of effect of coating the membranes in the Boyden Chambers with ECM proteins was unexpected, as cells expressing uPAR adhere to ECM by binding to ECM proteins such as VN and this interaction is necessary for the resulting cytoskeletal changes leading towards cell migration[171]. In instances where there is no direct interaction between uPAR and VN, uPA can lead to similar uPAR dependent cytoskeletal changes independent of VN[172]. It is possible that in the in-vitro environment of the Boyden Chamber, these cells either do

not express the relevant proteins (as the HEKa cells were from healthy adult) or lack the required assistance from ECM to induce a migratory response. It is also possible that the SRSRY peptide may be an important chemotactic factor but within the complex wound environment it may be degraded to an inactive form by proteases found within a wound environment. Evidence to support this possibility would need further detailed studies to identify and inhibit in-vivo proteases that may degrade suPARII-III

The results of the scratch assays showed that HEKa cells could not survive in the presence of high concentrations of ulcer exudates (both healing and non-healing) in vitro. This suggests the presence of an inhibitory substance(s) within these exudates. At lower concentrations healing exudates stimulated HEKa coverage of scratch compared with non-healing exudates. Our results are in contrast with a previous study where keratinocytes survived after treatment with 2% chronic wound exudates for 24 hours[176]. A possible explaination for this difference may be that we diluted the wound exutades in serum free media in order to see the true effect of chronic wound exudates where as in the previously done study the authors used growth meida to dilute the chronic wound exudates[176]. This would have provided the keratinocytes with growth supplements that may have prolonged the survival despite the presence of substances within the chronic wound exudates that would inhibit survival.

Treatment with mitomycin C did not significantly alter scratch coverage induced by either healing or non-healing ulcer exudates, suggesting a role for cellular migration as opposed to proliferation in the response to treatment with exudates. The death of HEKa in the presence of suPAR-depleted exudates also

suggests an important role for suPAR in survival of keratinocytes cells.

Chapter 7: General discussion and future work

The exact mechanisms leading from venous hypertension to venous leg ulceration still remain unclear. We have proven the presence of suPAR fragments within the environment of venous ulcers. We have also attempted to clarify the role of suPAR in venous ulceration as previous studies have shown increased levels of plasminogen components within venous ulcers when compared with normal skin[112, 177, 178]. In contrast to pervious studies we compared levels of suPAR fragments and other components of plasminogen system between healing and non-healing venous ulcers. Amongst the components of the plasminogen system that were examined suPAR appeared to be the only difference between healing and non-healing ulcers. Levels of suPARI-III and suPARII-III were significantly higher in exudates obtained from healing venous ulcers compared to non-healing venous ulcers. This difference remained significant irrespective of normalizing the level of suPAR according to volume (ng/ml) or soluble protein (ng/mg soluble protein).

No difference was observed in the levels of uPA and PAI-1 when healing and non-healing venous ulcers were compared. The lack of uPA activity in ulcer exudates, suggests either the presence of an inhibitory protease or the fact that uPA may only have an active role in cellular environment.

In a previous study treatment of keratinocytes and fibroblasts with 2% chronic wound exudate is shown to stimulate focal uPAR expression[176]. In our

experience 2% chronic wound exudate resulted in cell death. We attempted to augment the known role of uPAR by treating scratched in-vitro keratinocytes with healing ulcer exudates which were known to have a significantly higher level of suPAR compared with non-healing ulcer exudates. Scratch coverage was better in healing exudates (1% and 0.5%) when compared with non-healing ulcer exudates (1% and 0.5%). Although the scratch healing potential of the healing ulcer exudates cannot be completely attributed to suPAR, it can be concluded that suPAR had an important role in the migration and survival of keratinocytes as suggested by the results performed in the presence of Mytomicin C and suPAR-depleted healing exudates.

Validation of the assays could not be achieved for tissue homogenates. It may be due to the fact that the extraction buffer used may not be ideal for ulcer tissue. A number of other extraction buffers have been used[148] and attempting to use the various buffers in order to achieve a purified extraction of uPAR may help future studies. Patients with venous ulcers may be reluctant to allow for tissue biopsies as we found during our study and due to this we did not attempt to use the small tissue samples for different types of buffers.

The validation of suPAR for ulcer exudates was an important component of our study and will help future work for detection of suPAR forms in wound exudates. Although we did not find any correlation in suPAR levels of blood plasma and venous ulcer exudates yet the validation of specific antibodies in TR-FIAs for detecting various forms of suPAR may help future investigative wound studies.

Depletion of suPAR done in our study using affinity column chromatography resulted in depletion of all suPAR forms. Developing an antibody that can target the suPARII-III or suPARI form only can help clarify the specific roles of these fragments in keratinocyte migration and survival. In addition PAI-1 and vitronectin may influence suPAR functions and therefore antibodies that can block VN and/or PAI-1 may also elucidate the role of suPAR in ulcer healing.

Results from this study have shown that suPAR fragments may be important in ulcer healing. The precise mechanisms by which suPAR fragments can influence healing warrant further investigation. More importantly what needs to be determined now is which factors influence increased suPAR expression and cleavage within healing ulcers. These may be some of the proteases (MMPs) present within venous ulcers but detailed studies showing the precise role of such proteases resulting in increased suPAR cleavage when compared between healing and non-healing venous ulcers is required.

There are other contributing clinical factors that impair healing of a venous ulcer e.g high BMI, poor ankle mobility[193]. These factors were not focused upon during this study but it would be prudent to included these factors in future studies in order to get a realist influence of suPAR on venous ulcer healing.

The initial limitation to obtain only ten blood plasma samples by the ethics committee proved frustrating, as no meaningful outcome could be obtained for plasma analysis. Once the blood plasma results were analyzed a substantial amendment was submitted to the ethics committee but the delay in our validation work meant that many patients had already been started on

compression therapy by the time the first 10 plasma samples were analyzed. For future studies we hope there should be no limitation to the number of blood samples.

Finally a concesus needs to be reached on a single method for measuring proteins and cytokines within venous ulcer exudates. Standardization either according to volume or to soluble protein can reveal variable results. We have shown that standardizing the protein of interest according to volume or soluble protein content may have different results. Previous studies have measured proteins and cytokines within wound fluids in relation to volume (per ml)[182] and soluble protein (mg of soluble protein per ml). The reason to standardize measurements according to soluble protein is to nullify the effect of hydration status of patient, effect of heart failure and bacterial contamination[95]. Therefore a single agreed method would be beneficial to compare results from different centers.

Appendix A

Table for figure 32: Migration of THP-1 in response to chemotactic and scrambled peptides. Flourescent intensity with SEM (standard error of mean)

Time (mins)	SRSRY- 1pM	SRSRY – 1fM	C-pep – 1pM	C-pep – 1fM
0	0	0	0	0
20	5860±172	5969±436	5010±440	5504±189
30	7078±240	7400±758	5744±506	6226±178
60	10439±695	11197±340	8204±496	10425±725
90	11394±258	13035±685	8678±489	11304±725
120	14371±770	14224±390	9025±408	10794±687

Table for figure 33: Migration of HEKa in response of various concentration of chemotactic and scrambled peptides. Flourescent intensity with SEM (standard error of mean)

Time (mins)	SRSRY 1nM	SRSRY 1pM	SRSRY 1fM	SFM	C-pep 1nM	C-pep 1pM	C-pep 1fM
0	0	0	0	0	0	0	0
40	3244±265	6389±633	5132±283	4719±367	3089±194	4838±374	5212±218
100	2975±305	7773±742	5885±323	5043±482	2746±161	5378±499	5780±191
200	3030±171	8686±576	5658±198	4769±383	2898±233	4301±214	5008±148
280	3141±236	8554±786	5647±208	4736±247	2959±258	3683±220	4703±113
360	3005±197	7661±615	5501±222	4576±373	4223±376	3078±216	4622±138

Table for figure 34: Migration of HEKa in response to chemotactic peptide in presence of various ECM proteins. Flourescent intensity with SEM (standard error of mean)

Time (mins)	SRSRY no coat	SRSRY CN	SRSRY FN	SRSRY VN	C-pep no coat	C-pep CN	C-pep-FN	C-pep-VN
0	0	0	0	0	0	0	0	0
40	3041±42	3736±124	3835±287	3664±12	3071±37	3303±21	3068±398	3548±102
80	4141±38	4817±78	4530±388	4951±13	3258±19	3223±36	3656±387	3328±55
120	5199±147	6131±156	5839±389	6250±39	3307±78	3500±12	3902±493	3569±97
160	6566±97	7649±59	6473±527	7104±165	3131±62	4639±484	4048±578	3740±64
200	6429±106	6134±79	5824±316	6244±12	3066±39	3325±85	3500±417	3347±83

Table for figure 35: Migration of HEKa in response to various diultions of wound fluids. Flourescent intensity with SEM (standard error of mean)

Time (mins)	WF 2.5%	WF 5%	WF 10%	WF 15%	WF 20%
0	0	0	0	0	0
40	5652±183	5367±382	5247±92	5104±83	5644±40
100	7120±70	6876±288	7440±46	6763±89	6646±356
200	7249±126	6949±401	7033±124	6210±123	5822±1141
280	6803±121	6536±250	6122±210	5484±49	4794±1205
360	6423±211	6092±230	5780±98	5319±73	2868±376

Appendix B

Results from this work have been presented in the following conferences:

The role of soluble uPAR fragments in venous ulcer healing. **A Ahmad** – XVI world meeting of UIP (Union Internationale de Phlebology) – 30th August 2009 – Principality of Monaco – ***Awarded two year UIP Fellowship***

Reduced levels of soluble urokinase receptor (uPAR) fragment DII-III predicts venous ulcers that fail to heal. **Ahmad A**, Waltham M, Høyer-Hansen G, Sørensen TT, Mattock K, Saha P, Modarai B, Zayed H, Smith A. SARS 5th Jan 2011

Reduced expression of soluble urokinase receptor fragment DII-III predicts venous ulcers that fails to heal. **Ahmad A**, Waltham M, Høyer-Hansen G, Sørensen TT, Mattock K, Saha P, Modarai B, Zayed H, Smith A. 23rd Annual meeting Amercian Venous Forum. 24th Feb 2011

Reduced expression of soluble urokinase receptor fragment DII-III predicts venous ulcers that fail to heal. **A Ahmad**. International Union of Phlebology. 15th September 2011. Prague

Plasminogen activator receptor cleavage: an important mechanism in ulcer healing. **A Ahmad,** M Waltham, G Høyer-Hansen, TT Sørensen, K Mattock, A Patel, P Saha, J Humphries, C Evans, S Premaratne, B Modarai, A H Davies, H Zayed, A Smith. Vascular Society, 23rd November 2011. Edinburgh, UK

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